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Molecular Genetics of Aminoglycoside Resistance Genes and Familial Relationships of the Aminoglycoside-Modifying Enzymes

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INTRODUCTION 139
CHARACTERIZATION OF AMINOGLYCOSIDE-MODIFYING ENZYMES 139
Acetylation 139
AAC(1) 139
AAC(3) 140
(i) AAC(3)-I 140
(ii) AAC(3)-II 141
(iii) AAC(3)-III 144
(iv) AAC(3)-IV 144
(v) AAC(3)-VI 144
(vi) AAC(3)-VII, AAC(3)-VIII, AAC(3)-IX, and AAC(3)-X 144
AAC(6′) 144
(i) AAC(6′)-I 144
(ii) AAC(6′)+APH(2″) 145
(iii) AAC(6′)-II 145
AAC(2′) 145
(i) AAC(2′)-I 145
Adenylylation 145
ANT(2″) 145
(i) ANT(2″)-I 145
ANT(3″) 145
(i) ANT(3″)-I 145
ANT(4′) 145
(i) ANT(4′)-I 145
(ii) ANT(4′)-II 145
ANT(6) 146
(i) ANT(6)-I 146
ANT(9) 146
(i) ANT(9)-I 146
Phosphorylation 146
APH(3′) 146
(i) APH(3′)-I 146
(ii) APH(3′)-II 146
(iii) APH(3′)-III 146
(iv) APH(3′)-IV 146
(v) APH(3′)-V 146
(vi) APH(3′)-VI 147
(vii) APH(3′)-VII 147
APH(3″) 147
(i) APH(3″)-I 147
APH(6) 147
(i) APH(6)-I 147
APH(4) 147
(i) APH(4)-I 147
STRUCTURE-FUNCTION RELATIONSHIPS AMONG FAMILIES OF AMINOGLYCOSIDE-MODIFYING ENZYMES 147
AAC(6′) Family of Proteins 149
AAC(3) Family of Proteins 151

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AAC(2') Protein 152
Phosphorylating Family of Proteins 152
APH(3')-I 154
APH(3')-II 154
APH(3')-V 155
APH(3")-I and APH(6)-I 155
APH(4)-I 155
Adenylylating Family of Proteins 155
ANT(2")-I 155
ANT(3")-I and ANT(9)-I 155
ANT(4')-I and ANT(4')-II 155
ANT(6)-I 156
ORIGIN AND MECHANISMS OF DISSEMINATION OF THE GENES ENCODING AMINOGLYCOSIDE-MODIFYING ENZYMES 156
REGULATION OF AMINOGLYCOSIDE RESISTANCE GENES 156
CELLULAR LOCALIZATION OF AMINOGLYCOSIDE-MODIFYING ENZYMES 159
CONCLUSIONS AND PROSPECTS 159
REFERENCES 160

INTRODUCTION

In bacteria, resistance to aminoglycosides is often due to enzymatic inactivation by acetyltransferases, nucleotidyltransferases (adenylyltransferases), and phosphotransferases (5, 24). Other mechanisms include ribosomal alterations and loss of permeability, which have been reviewed previously (5, 24) and will not be discussed here. Aminoglycoside-resistant strains often emerge as a result of acquiring plasmid-borne genes encoding aminoglycoside-modifying enzymes (16). Furthermore, many of these genes are associated with transposons, which aid in the rapid dissemination of drug resistance across species boundaries.

The DNA sequences of many genes encoding aminoglycoside-modifying enzymes have been determined. DNA hybridization studies, using probes developed from these genes, have been crucial for understanding the origin, frequency, and dissemination of these genes. With the advent of large-scale DNA hybridization techniques, we can now study the following questions concerning specific genes. Are resistance genes restricted to specific species? What are the factors that aid or limit the spread of aminoglycoside resistance genes? What is the significance of clinical isolates which contain multiple resistance genes?

Comparison of the predicted amino acid sequences of the aminoglycoside-modifying enzymes allows an assessment of the relationships between enzymes with different resistance profiles. Some analysis by identification of aminoglycoside-resistant mutants with alterations in protein sequence and changes in the resistance profiles has been performed (8, 44, 77, 112). The data generated from these mutant studies and from protein sequence homology studies have allowed gross modeling of the molecular interactions between aminoglycosides and the resistance enzymes, with an assessment of the amino acids which may be important in binding aminoglycosides. From this work it may be possible to predict the changes that are necessary for development of new resistance spectra and the speed with which they can arise.

Studies of the derivation of aminoglycoside resistance genes have suggested that they originated from both producing organisms and mutation of normal cellular genes (5, 73). Our recent studies have provided evidence that the regulation of normally quiescent cellular genes can be altered so that high-level expression can lead to aminoglycoside resistance (91).

CHARACTERIZATION OF AMINOGLYCOSIDE-MODIFYING ENZYMES

A total of 4,228 clinical isolates were collected between 1987 and 1991 (60a). The isolates were chosen on the basis of resistance to one or more aminoglycosides, and duplicate strains were eliminated. The bacteria were divided into six groups: (i) gram-negative bacteria which are usually susceptible to aminoglycosides, excluding (ii) *Pseudomonas* spp., (iii) *Serratia* spp., (iv) *Acinetobacter* spp., and (v) miscellaneous gram-negative bacteria belonging to rare genera or which are usually aminoglycoside resistant, and (vi) *Staphylococcus* spp. The strains were isolated in Belgium, France, the Netherlands, Luxembourg, Germany, Greece, Italy, Argentina, Chile, Guatemala, Uruguay, Mexico, and Venezuela.

A summary of the data on the frequency of aminoglycoside resistance profiles (AGRPs) in this group of strains and a summary of all of the known genes encoding aminoglycoside resistance enzymes are shown in Table 1. The nomenclature used is defined as follows: AAC (acetyltransferase), ANT (nucleotidyltransferase or adenylyltransferase), and APH (phosphotransferase) for the type of enzymatic modification; (1), (3), (6), (9), (2'), (3'), (4'), (6'), (2"), and (3") for the site of modification; I, II, II, IV, V, etc. for unique resistance profiles; and a, b, c, etc., for unique protein designations. Therefore, AAC(6')-Ia and AAC(6')-Ib are two unique proteins conferring identical resistance profiles. The nomenclature of the genes which encode these enzymes is a modification of the genotype nomenclature of Mitsuhashi (62); e.g., *aac(6')-Ia* and *aac(6')-Ib* are unique genes encoding two proteins with the same resistance profile.

Acetylation

Four classes of *N*-acetyltransferases, which modify aminoglycosides in the 1-, 3-, 6'-, and 2'-amino groups, have been identified (5, 24, 55, 62).

AAC(1). The AAC(1) AGRP is characterized by resistance to apramycin, lividomycin, paromomycin, and ribostamycin (32, 55). In addition, in vitro enzyme assays demonstrated that butirosin and neomycin were acetylated by this enzyme (55) (Table 1). The AAC(1) enzyme was produced by *Escherichia coli* J62-1, which was one of five apramycin-resistant veterinary isolates (55). The gene encoding this enzyme has not been cloned, nor has the distribution of this AGRP been examined.

TABLE 1. Characteristics of aminoglycoside-modifying enzymes

Resistance mechanism	Cloned genes	Alternative nomenclature	GenBank accession no.	Aminoglycoside resistance profile[a]	Mol. mass (kDa) (D/P)[b]	pI value (P)[c]
Acetylation						
AAC(1)				Apr, Lvdm, Prm, Rsm, (But), (Neo)		
AAC(3)-I				Gm, Astm, Siso		
	aac(3)-Ia	*aacC1*	X15852		19 D/19.4 P	6.12
	aac(3)-Ib		L06157		19.3 P	5.31
AAC(3)-II				Gm, Tob, Dbk, Ntl, 6'Ntl, 2'Ntl, Siso		
	aac(3)-IIa	*aacC3*, *aacC5*, *aacC2*, *aac(3)-Va*	X13543		31.5 D/30.5 P	6.43
	aac(3)-IIb	*aac(3)-Vb*	M97172		29.6 P	5.11
	aac(3)-IIc	*aacC2*	X54723		30.5 P	6.42
AAC(3)-III				Gm, Tob, Dbk, 5-epi, Siso, Km, Neo, Prm, Lvdm		
	aac(3)-IIIa	*aacC3*	X55652		29.6 P	5.52
	aac(3)-IIIb		L06160		29.0 P	4.84
	aac(3)-IIIc	*ant(2")-Ib*	L06161		29.6 P	4.88
AAC(3)-IV				Gm, Tob, Dbk, Ntl, 6'Ntl, 2'Ntl, Apr, Siso		
	aac(3)-IVa		X01385		28.5 D/29.2 P	5.83
AAC(3)-VI				Gm, 6'Ntl, Siso, (Tob), (Ntl), (5-epi), (Km)		
	aac(3)-VIa		M88012		32.1 P	6.61
AAC(3)-?				?		
AAC(3)-VII	*aac(3)-VIIa*	*aacC7*	M22999		31.1 P	4.96
AAC(3)-VIII	*aac(3)-VIIIa*	*aacC8*	M55426		30.4 P	6.06
AAC(3)-IX	*aac(3)-IXa*	*aacC9*	M55427		31.0 P	5.96
AAC(3)-X	*aac(3)-Xa*		D00681		30.8 P	5.93
AAC(6')-I				Tob, Dbk, Ntl, Amk, 2'Ntl, 5-epi, Siso, (Isp)		
	aac(6')-Ia	*aacA1*	M18967		21.3 P	5.18
	aac(6')-Ib	*aacA4*	M21682		24.5 D/22.4 P	5.01
	aac(6')-Ic		M94066		16.3 P	5.29
	aac(6')-Id		X12618		16.8 P	5.11
	aac(6')-Ie	*aac(6')*-bifunctional	M18086	Plus Astm	[23.5]	[5.14]
	aac(6')-If		M55353		16.0 P	4.99
	aac(6')-Ig					
	aac(6')-Ih					
	aac(6')-Ii					
AAC(6')-II				Gm, Tob, Dbk, Ntl, 2'Ntl, 5-epi, Siso		
	aac(6')-IIa		M29695		20.7 P	4.79
	aac(6')-IIb		L06163		19.8 P	4.84
AAC(6')-III				2'Ntl only [low level AAC(6')-I activity]		
	aac(6')-Ic	See AAC(6')-Ic				
AAC(6')-APH(2")				Gm, Tob, Dbk, Ntl, Amk, 2'Ntl, 6'Ntl, 5-epi, Astm		
	aac(6')-aph(2")		M18086, M13771		56 D/56.8 P	4.1
AAC(2'-I)				Gm, Tob, Dbk, Ntl, 6'Ntl		
	aac(2')-Ia		L06156		20.1 P	5.09

AAC(3). The DNA and deduced protein sequences of 14 genes encoding at least five distinct AAC(3) resistance patterns were previously determined (Table 1).

(i) AAC(3)-I. The AAC(3)-I AGRP is characterized by resistance to gentamicin and fortimicin (Astromicin) (11, 92). This AGRP is widespread among members of the family *Enterobacteriaceae* and was found in 10 to 17% of gram-negative, *Pseudomonas* and *Serratia* strains, as well as 29.6% of *Acinetobacter* strains examined (Table 1). The DNA sequences of two *aac(3)-I* genes have been determined: *aac(3)-Ia* (98, 99, 109) and *aac(3)-Ib* (86). DNA hybridization studies demonstrated that the *aac(3)-Ia* gene was found in

TABLE 1—*Continued*

Cloned from	Plasmid/chromosome	Mechanism distribution (%)[d]					% Probe positive[e]	Reference(s)
		Gm-neg.	Pseudo.	Serr.	Acineto.	Staph.		
								55
		10.8	10.6	16.8	29.6			
E. coli SCH72091801	pJR88						82	98, 100, 109
P. aeruginosa STONE130								86
		60.3	18	32.4	21.3			
S. marcescens	pCER954b/pWP113a						84.8	1, 10, 106
S. marcescens							5.9	76
E. coli								105
P. aeruginosa PST-1								107
P. aeruginosa SCH82122811								91a
P. aeruginosa								51, 87a
		3.5	0.5	0.6	0.5			
Salmonella sp.	pWP7b							9, 10, 14, 19
		0.2	1.2					
E. cloacae	pSCH20217						50	70, 75
S. rimosus								54, 70a
S. fradiae								81
M. chalcea								81
S. griseus SS-1198PR	Chromosomal							39a, 40
		47.8	9.6	76.9				
C. diversus	pBWH100						0.1	96
S. marcescens	pAZ5007						70.6	64, 77, 102
S. marcescens	Chromosomal						21.3	13, 26, 42, 91
K. pneumoniae	Tn*4000*							83
(Gram-positive)								23, 91a
E. cloacae/*Citrobacter* spp.	pUO490							90a, 100
Acinetobacter haemolyticus								46a
Acinetobacter spp.								46a
Enterococcus spp.								50a
P. aeruginosa			48.4				90	77, 88
P. aeruginosa								74a
								91
						98.9		
S. faecalis, *S. aureus*	pIP800, Tn*4001*						53.9	23, 53, 79
P. stuartii	Chromosomal	5.8	0.1					78, 111

82% of all strains expressing an AAC(3)-I AGRP (Table 1), similar to the results of a previous survey (77.9%) (89). Work is currently in progress to determine the frequency of the *aac(3)-Ib* gene among AAC(3)-I strains.

(ii) AAC(3)-II. The AAC(3)-II AGRP is characterized by resistance to gentamicin, tobramycin, dibekacin, netilmicin, 2′-*N*-ethylnetilmicin, 6′-*N*-ethylnetilmicin, and sisomicin (92). This AGRP has been previously designated AAC(3)-V (2, 76, 89). Since DNA sequence analysis has shown that the genes encoding the AAC(3)-II and AAC(3)-V resistance profiles are identical (1, 2, 106), in the interest of uniformity we have changed the designation of this resistance profile to

TABLE 1—*Continued*

Resistance mechanism	Cloned genes	Alternative nomenclature	GenBank accession no.	Aminoglycoside resistance profile[a]	Mol. mass (kDa) (D/P)[b]	pI value (P)[c]
Adenylylation						
ANT(2″)-I				Gm, Tob, Dbk, Siso, Km		
	ant(2″)-Ia	*aadB*	X04555		28.0 P	4.73
	ant(2″)-Ib	*aac(3)-IIIc*	L06161		29.6 P	4.88
	ant(2″)-Ic					
ANT(3″)-I				Sm, Spcm		
	ant(3″)-Ia	*aadA*, *aad(3″)(9)*	X02340, M10241		31.6 D/33.2 P	4.68
ANT(4′)-I				Tob, Amk, Isp, Dbk		
	ant(4′)-Ia		V01282		34 D/29.1 P	4.7 D/4.6P
ANT(4′)-II				Tob, Amk, Isp		
	ant(4′)-IIa		M98270		29.2 P	4.55
ANT(6)-I				Sm		
	ant(6)-Ia	*ant6*, *aadE*			36.1 P	4.72
ANT(9)-I				Spcm		
	ant(9)-Ia	*aad(9)*, *spc*	X13290		29.0 P	8.38
Phosphorylation						
APH(2″)-I				See AAC(6′)-APH(2″)		
	aph(2″)-Ia	*aph(2″)*-bifunctional			[35.8 P]	[4.38]
APH(3′)-I				Km, Neo, Prm, Rsm, Lvdm, GmB		
	aph(3′)-Ia	*aphA-1*	J01839		31.0 P	5.08
	aph(3′)-Ib	*aphA*-like	M20305		30.1 P	4.91
	aph(3′)-Ic	*apha1-IAB*, *apha7*	M37910		30.9 P	5.41
APH(3′)-II				Km, Neo, Prm, Rsm, But, GmB (Amk)		
	aph(3′)-IIa	*aphA-2*	V00618		27 D/29.2 P	4.48
APH(3′)-III				Km, Neo, Prm, Rsm, Lvdm, But, GmB, Amk, Isp		
	aph(3′)-IIIa		V01547		31 D/30.6 P	4.1 D/4.37 P
APH(3′)-IV				Km, Neo, Prm, Rsm, But		
	aph(3′)-IVa	*aphA4*	X01986		28.5 D/29.9 P	4.8
APH(3′)-V				Neo, Prm, Rsm		
	aph(3′)-Va	*aphA-5a*	K00432		32 D/30.0 P	4.46
	aph(3′)-Vb	*aphA-5b*, *rph*	M22126		29.5 P	4.5
	aph(3′)-Vc	*aphA-5c*			29.8 P	4.44
APH(3′)-VI				Km, Neo, Prm, Rsm, But, GmB, Amk, Isp		
	aph(3′)-VIa	*aphA6*	X07753		30.3 P	4.43
	aph(3′)-VIb					
APH(3′)-VII				Km, Neo (Amk)		
	aph(3′)-VIIa	*aphA7*	M29953		29.7 P	4.58
APH(3″)-I				Sm		
	aph(3″)-Ia	*aphE*, *aphD2*	X53527		29.0 P	4.83
	aph(3″)-Ib	*strA*, *orfH*	M28829		29.6 P	4.77
APH(6)-I				Sm		
	aph(6)-Ia	*aphD*, *strA*	Y00459		33.2 P	4.48
	aph(6)-Ib	*sph*	X05648		33.1 P	4.51
	aph(6)-Ic	*str*	X01702		29.0 P	4.97
	aph(6)-Id	*strB*, *orfI*	M28829		30.8 P	4.73
APH(4)-I				HygB		
	aph(4)-Ia	*hph*	V01499		41 D/38 P	4.66
	aph(4)-Ib	*hyg*	X03615		60 D/37 P	4.48

[a] Abbreviations: Amk, amikacin; Apr, apramycin; Astm, Astromicin (fortimicin); But, butirosin; Dbk, dibekacin; 5-epi, 5-episisomicin; Gm, gentamicin; GmB, gentamicin B; HygB, hygromycin B; Isp, isepamicin; Km, kanamycin; Lvdm, lividomycin; Neo, neomycin; Ntl, netilmicin; 2′Ntl, 2′-*N*-ethylnetilmicin; 6′Ntl, 6′-*N*-ethylnetilmicin; Sm, streptomycin; Prm, paromomycin; Rsm, ribostamycin; Siso, sisomicin; Spcm, spectinomycin; Tob, tobramycin. Parentheses indicate that although resistance was not conferred, enzymatic activity was detectable in vitro. In some cases, MICs of these compounds were slightly elevated. It is possible that strains containing additional mutations which alter the general uptake of aminoglycosides (permeability) show high-level resistance to these compounds, as has been previously demonstrated for amikacin resistance medicated by APH(3′)-IIa (72).

[b] Abbreviations: D, determined molecular mass; P, molecular mass predicted from putative protein sequence data.

[c] Abbreviation: P, predicted pI; D, experimentally determined pI value.

[d] The total of 4,228 strains examined included 728 *Pseudomonas* strains (Pseudo.), 494 *Serratia* strains (Serr.), 206 *Acinetobacter* strains (Acineto.), 262

TABLE 1—*Continued*

Cloned from	Plasmid/chromosome	Mechanism distribution (%)[d]					% Probe positive[e]	Reference(s)
		Gm-neg.	Pseudo.	Serr.	Acineto.	Staph.		
		14.9	18.4	21.1	15.5			
Enterobacteriaceae	pSCL14						87	37
K. oxytoca, strain 80	pSCL29							52, 91a
E. cloacae, strain 178	pSCL35							52
Enterobacteriaceae	R 538-1							37
						30.2		
S. aureus							84.8	59, 82, 87
		0.2	0.1					
P. aeruginosa	pMG77						0	41, 90
E. faecalis	pJH1							67
S. aureus	Tn*554*							63
								23
		46	6.6	27.5	49.5			
K. pneumoniae	Tn*903*						90.5	65
	RP4							69
K. pneumoniae	pBWH77							51
		0.6	3.1					
S. typhimurium	Tn*5*						2.5	3, 8, 44, 71, 72, 112
S. aureus/*S. faecalis*	pAT4/pJH1							27, 49, 103
B. circulans NRRL3312	Chromosomal							35
S. fradiae ATCC10745	Chromosomal							101
S. ribosidificus SF733	Chromosomal							39
M. chalcea 69-683	Chromosomal							80
		0.8	1.6	0.2	35.4			
A. baumannii	pIP1841						82.7	47, 48, 57
K. pneumoniae	pRPG101							25
C. jejuni PS1178	14-kb plasmid							95, 97
S. griseus N2-3-11								34
	RSF1010							84
S. griseus N2-3-11								56
S. glaucescens								108
	Tn*5*							60
	RSF1010							84
E. coli W677	pJR225							28
S. hygroscopicus								28, 113

Staphylococcus strains (Staph.), 93 miscellaneous gram-negative bacteria that belong to rare genera or that are usually aminoglycoside resistant (the data for this group are not shown), and 2,445 other gram-negative bacteria (Gm-neg.). The percent mechanism distribution is given by the number of strains which express an AGRP divided by the number of strains in the bacterial group [e.g., 18.4% of the *Pseudomonas* strains expressed an ANT(2")-I AGRP].

[e] The percent probe positive is given by the number of strains which hybridized to the probe divided by the total number of strains which expressed the AGRP [e.g., 87% of all strains expressing an ANT(2")-I resistance profile hybridized to the *ant(2")-Ia* probe]. Hybridization conditions and most of the DNA probes were as previously described (89). Additional probes included *aac(3)-IIb* (76); *aac(3)-VIa* (75); *aac(6')-Ic* (91); *aac(6')+aph(2")*, the 616-bp *Hpa*I-*Sca*I fragment, which includes only the *aac(6')-Ie* portion of the bifunctional gene (23); *ant(4')-Ia* (90); *ant(4')-IIa* (90); and *aph(3')-VIa*, the 370-bp *Aac*I-*Eco*RI fragment from pAT240 (48, 57).

AAC(3)-II. The AGRP is very commonly observed in members of the *Enterobacteriaceae*; however, the data in Table 1 indicate that the frequency varies among different genera: 60.3% of gram-negative bacteria, 18% of *Pseudomonas* spp., 32.4% of *Serratia* spp., and 21.3% of *Acinetobacter* spp. The DNA sequences of three genes encoding this AGRP have been determined. The *aac(3)-IIa* gene (1, 2, 99, 106) is the most common, being present in 84.8% of isolates which express this AGRP. The sequence of another gene [*aac(3)-IIc*] was 97% identical to that of *aac(3)-IIa*, with changes in only 26 bp resulting in 12 amino acid substitutions (105). It is very likely that the *aac(3)-IIa* probe would hybridize to strains carrying the *aac(3)-IIc* gene. A third gene [*aac(3)-IIb*] which expressed an AAC(3)-II profile but did not hybridize to the *aac(3)-IIa* probe was cloned from *Serratia marcescens* (76). This gene was 72% identical to the *aac(3)-IIa* gene. The *aac(3)-IIb* gene was found in 5.9% of the clinical isolates which expressed an AAC(3)-II AGRP (Table 1).

(iii) AAC(3)-III. The AAC(3)-III AGRP is characterized by resistance to gentamicin, tobramycin, dibekacin, and 5-episisomicin (7). Three genes encoding the AAC(3)-III AGRP have been cloned, one from *Pseudomonas aeruginosa* Travers [*aac(3)-IIIa*] (107) and two from other *Pseudomonas* strains (50, 91a).

Comparison of the DNA sequences of *ant(2")-Ib* (87a) and *aac(3)-IIIc* (50) showed that they are identical, beginning at least 50 bp before the coding sequence and extending to at least 100 bp beyond the coding sequence. The protein encoded by this sequence is also 92% similar to the predicted sequence of the AAC(3)-IIIb protein (91a) and 66% similar to the AAC(3)-IIIa protein from *P. aeruginosa* Travers (107). Since the difference in the resistance spectrum between the ANT(2")-I and AAC(3)-III AGRPs is the ability to modify 5-episisomicin, we have compared the resistance profiles of strains containing the cloned *ant(2")-Ia*, *ant(2")-Ib*, *aac(3)-IIIb*, and *aac(3)-IIIc* genes. We have found that the AGRP resulting from the *ant(2")-Ib* gene is identical to the AAC(3)-III profile in that a low level of 5-episisomicin resistance is observed. This resistance to 5-episisomicin is not observed in strains carrying the *ant(2")-Ia* gene. Recent experiments demonstrated that the *ant(2")-Ib/aac(3)-IIIc* gene encodes an enzyme which acetylates gentamicin, tobramycin, and 5-episisomicin and has no adenylylating or phosphorylating activity (26a). Therefore, we believe that the *ant(2")-Ib* gene cloned from plasmid pSCL29 was misclassified (52) and that it actually encodes an *aac(3)-III* gene.

(iv) AAC(3)-IV. The AAC(3)-IV AGRP is characterized by resistance to gentamicin, tobramycin, dibekacin, netilmicin, 2'-*N*-ethylnetilmicin, 6'-*N*-ethylnetilmicin, apramycin, and sisomicin (19). This AGRP was rarely observed in members of the *Enterobacteriaceae*. The *aac(3)-IVa* gene was cloned from a *Salmonella* veterinary isolate (10) and was shown to have transferred to clinically important organisms (14). In our recent study, this AGRP was found in 3.5% of the gram-negative organisms (Table 1). The gene encoding hygromycin resistance (*hyg*) was found downstream of the *aac(3)-IVa* gene and was cotranscribed with it, utilizing the IS*140* promoter (9).

(v) AAC(3)-VI. The AAC(3)-VI AGRP is characterized by resistance to gentamicin and 6'-*N*-ethylnetilmicin (70). Although resistances to tobramycin, netilmicin, 6'-*N*-ethylnetilmicin, 5-episisomicin, and kanamycin were not conferred, a low level of enzymatic activity against these compounds was detected (70). This AGRP is extremely rare among members of the *Enterobacteriaceae* (Table 1). However, DNA hybridization studies have shown that the *aac(3)-VIa* probe (75) hybridized to 65 strains (5.7%) which did not express an AAC(3)-VI AGRP. These strains included 27 *E. coli*, 17 *Pseudomonas*, 6 *Enterobacter*, and 5 *Klebsiella* strains. This high rate of false-positives may be due to hybridization to a cryptic chromosomal gene which contains DNA sequences homologous to the probe. This chromosomal gene may represent the ancestral gene from which *aac(3)-VIa* was derived.

(vi) AAC(3)-VII, AAC(3)-VIII, AAC(3)-IX, and AAC(3)-X. Four additional *aac(3)* genes have been cloned from actinomycetes strains (54, 80, 81). Table 1 lists the resistance mechanisms encoded by these genes as AAC(3)-VII, AAC(3)-VIII, AAC(3)-IX, and AAC(3)-X. López-Cabrera et al. (54) reported that the *aac(3)-VIIa* (*aacC7*) gene gave rise to a substrate profile which was nearly indistinguishable from the AAC(3)-II AGRP. However, a direct comparison of the AAC(3)-II and AAC(3)-VII substrate profiles was not shown (70a). In addition, since the aminoglycoside resistance profiles were not tested, it cannot be determined whether the resistance profile would be identical to AAC(3)-II if a complete spectrum of aminoglycosides were used. Similarly, the AAC(3)-X resistance profile and substrate profile included gentamicin, dibekacin, kanamycin, and to a lesser extent neomycin, and paromomycin (39a). Since it is difficult to compare the resistance profiles of these four enzymes, they were assigned to the AAC(3)-? class (Table 1).

Where tested, these actinomycetes genes were not expressed in *E. coli*. Work is in progress to examine the resistance profile of the *aac(3)-VIIa* gene in *E. coli* by using the *lacZ* promoter to test whether this gene encodes an AAC(3)-II-type enzyme (87a).

AAC(6'). A third class of acetylating enzymes modifies the 6'-amino group of aminoglycosides. Some of these enzymes are capable of modifying the clinically important aminoglycosides tobramycin, netilmicin, amikacin, fortimicin, sisomicin, and gentamicin C_{1a} and C_2 but are less capable of modifying gentamicin C_1 and isepamicin.

(i) AAC(6')-I. The AAC(6')-I AGRP is characterized by resistance to tobramycin, dibekacin, amikacin, 5-episisomicin, netilmicin, 2'-*N*-ethylnetilmicin, and sisomicin (92). The observed frequency of this AGRP varied among different organisms: 47.8% of gram-negative bacteria, 9.6% of *Pseudomonas* spp., and 76.9% of *Serratia* spp. (Table 1). At least six genes which encode this AGRP have been identified: *aac(6')-Ia* (96) was extremely rare (0.1%); *aac(6')-Ib* (64, 102) was the most prevalent, found in 70.6% of strains expressing this AGRP; *aac(6')-Ic* (91) was found in 21.3% of AAC(6')-I strains; *aac(6')-Id* (83, 91) was extremely rare (see below); *aac(6')-Ie* (23, 79), which encodes the amino-terminal portion of the bifunctional enzyme AAC(6')+APH(2"), was found only in gram-positive bacteria (see below); and *aac(6')-If* (100) was cloned from *Enterobacter cloacae*. In addition to these six genes, several other *aac(6')-I* genes have recently been cloned: *aac(6')-Ig* and *aac(6')-Ih* from *Acinetobacter* spp. (46a); and *aac(6')-Ii* from *Enterococcus faecium* (15).

The *aac(6')-Ic* gene was cloned from *S. marcescens* (13), and DNA hybridization analysis demonstrated that all *S. marcescens* strains carried the *aac(6')-Ic* gene, whether or not the AAC(6')-I resistance profile was expressed (91). DNA sequence analysis showed at least two "chromosomal sequences" adjacent to the *aac(6')-Ic* gene: the *rpoD* gene and isoleucine *tRNA-2* (91). These data were consistent with the chromosomal location of this gene in *Serratia* strains (13,

26, 42). DNA hybridization to 3,842 non-*Serratia* strains showed that the *aac(6')-Ic* gene was never found in organisms other than *Serratia* spp. (91).

The putative *aac(6')-Id* gene is an open reading frame located on Tn*4000* (83, 91, 100). This gene appears to be uncommon. A total of 238 organisms which demonstrated an AAC(6')-I profile were probed with an oligonucleotide made to the presumptive *aac(6')-Id* gene. Included were strains which did not hybridize with the *aac(6')-Ia*, *aac(6')-Ib*, and *aac(6')-Ic* probes. The *aac(6')-Id* probe failed to hybridize to any strain tested (87a).

(ii) AAC(6')+APH(2"). The AAC(6')+APH(2") AGRP is characterized by resistance to gentamicin, tobramycin, dibekacin, netilmicin, 2'-*N*-ethylnetilmicin, 6'-*N*-ethylnetilmicin, amikacin, isepamicin, 5-episisomicin, and fortimicin (53, 79, 92). This protein is composed of two separable domains. We and others (23) have expressed the AAC(6') amino-terminal domain and demonstrated that it encodes an AAC(6')-I resistance profile. In addition, we have found that the amino-terminal portion expresses resistance to fortimicin (91a). We have designated this gene portion *aac(6')-Ie*. The carboxy-terminal portion has protein sequence homology with other aminoglycoside-phosphorylating enzymes and has been shown to encode APH(2") activity (23).

A recent DNA hybridization study characterized 416 strains of staphylococci and enterococci, isolated from 24 hospitals in France, as expressing an AAC(6')+APH(2") mechanism (68). This study showed that 100% of the AAC(6')+APH(2") strains hybridized to the *aac(6')+aph(2")* probe. However, the data in Table 1 show that although 98.9% of the *Staphylococcus* strains tested in the present study have this phenotype, only 53.9% hybridized to the *aac(6')+aph(2")* probe. The discrepancy in the percentage of probe-positive strains may be due to differences in hybridization techniques. In the first study, cells were converted to spheroplasts with lysostaphin and lysed, and total DNA was spotted onto nitrocellulose filters for hybridization (68). In the current study, cells were spotted on GeneScreen Plus paper and treated with 0.5 M NaOH. Poor lysis of gram-positive strains with NaOH may have ultimately resulted in failure to hybridize with the *aac(6')+aph(2")* probe. It is therefore premature to speculate whether more than one gene encodes the AAC(6')+APH(2") resistance profile.

The bifunctional AAC(6')+APH(2") mechanism is thought to be restricted to gram-positive organisms. However, Kettner et al. (43) have recently reported the occurrence of this enzyme in gram-negative bacteria. If confirmed, these data would be significant and would demonstrate the ability of a plasmid-encoded gram-positive enzyme to be transferred to and expressed in gram-negative bacteria.

(iii) AAC(6')-II. The AAC(6')-II AGRP is characterized by resistance to gentamicin, tobramycin, dibekacin, netilmicin, 2'-*N*-ethylnetilmicin, and sisomicin (92). This AGRP has been observed only in *Pseudomonas* strains (61). DNA sequence analysis has demonstrated 74% sequence identity between the *aac(6')-Ib* gene and the *aac(6')-IIa* gene of *P. aeruginosa* (88), resulting in cross-hybridization between the *aac(6')-Ib* probe and AAC(6')-IIa strains (91). However, an *aac(6')-Ib* probe cross-hybridized with only 90% of *Pseudomonas* strains with an AAC(6')-II AGRP, suggesting that there is at least one other gene which encodes the AAC(6')-II AGRP. A second gene [*aac(6')-IIb*] which did not hybridize to the *aac(6')-Ib* probe has recently been cloned from a *Pseudomonas* strain (74a).

AAC(2'). **(i) AAC(2')-I.** The AAC(2')-I AGRP is characterized by resistance to gentamicin, tobramycin, dibekacin, netilmicin, and 6'-*N*-ethylnetilmicin (92, 111). This AGRP is restricted primarily to the *Providencia/Proteus* group of organisms. However, a few instances of this AGRP have been observed in *Pseudomonas* strains (0.1%) (29a). A chromosomal gene encoding this AGRP has recently been cloned from a *Providencia stuartii* strain (78).

Adenylylation

ANT(2"). **(i) ANT(2")-I.** The ANT(2")-I AGRP is characterized by resistance to gentamicin, tobramycin, dibekacin, sisomicin, and kanamycin (12). This AGRP is widespread among all gram-negative bacteria and was found in 14.9 to 21.1% of strains tested (Table 1). Three genes encoding 2"-*O*-adenylyltransferase activity have been reported (52). The *ant(2")-Ia* gene is the most common. It was observed in 87% of strains expressing ANT(2")-I (Table 1), similar to the frequency observed (77.6%) in a previous study by Shaw et al. (89). We have cloned and determined the DNA sequence of the *ant(2")-Ib* gene from pSCL29, isolated by Lee and coworkers (52, 91a). DNA sequence analysis demonstrated that the *ant(2")-Ib* gene is identical to the *aac(3)-IIIc* gene (see above). It is likely that the *ant(2")-Ib* gene was misclassified and is actually an *aac(3)-III* gene. Previous hybridization studies have shown that this gene is very rare (2.6%) (89). It is clear that an additional gene(s) is responsible for the remaining ~13 to 22% of the strains expressing an ANT(2")-I AGRP, which are *ant(2")-Ia* probe negative.

ANT(3"). **(i) ANT(3")-I.** The ANT(3")-I AGRP is characterized by resistance to streptomycin and spectinomycin (20, 37). The enzyme modifies the 3"-hydroxyl position of streptomycin and the 9-hydroxyl position of spectinomycin (20, 37). The *ant(3")-Ia* gene has been cloned in association with several transposons (37, 83) and is ubiquitous among gram-negative bacteria. In a previous study, 58.7% of the surveyed strains were streptomycin resistant (resistance to spectinomycin was not tested), and of these streptomycin-resistant strains, 55.5% carried the *ant(3")-Ia* gene (89).

ANT(4'). The ANT(4') aminoglycoside adenylyltransferases confer resistance to tobramycin, amikacin, isepamicin, and other aminoglycosides with 4'-hydroxyl groups (41).

(i) ANT(4')-I. The ANT(4')-I enzyme [also designated ANT(4',4")-I] has been shown to modify aminoglycosides at both the 4'- and 4"-hydroxyl groups, and, thus, resistance to dibekacin is also conferred (82, 87). This mechanism is restricted to gram-positive bacteria (61, 87). The ANT(4')-I mechanism was found in 30% of *Staphylococcus* strains tested, and the *ant(4')-Ia* probe hybridized to 84.8% of gram-positive strains which contained this phenotype (Table 1). The failure of strains to hybridize to the *ant(4')-Ia* probe may have been due to incomplete lysis of gram-positive bacteria with 0.5 M NaOH or may indicate that more than one gene encodes the ANT(4')-I resistance profile.

(ii) ANT(4')-II. The ANT(4')-II mechanism first appeared in 1981 in a Denver Veterans Administration hospital following an outbreak of amikacin resistance in gram-negative bacteria. It was first observed in isolates of *P. aeruginosa*, but by 1984 it had been observed in *E. coli*, *Citrobacter* spp., *Klebsiella* spp., and *Serratia* spp. (41). Clinical isolates with an ANT(4')-II AGRP failed to hybridize with an *ant(4')-Ia* probe (41). These results suggested that the gene found in gram-positive bacteria was different from the gene encoding the enzyme observed in gram-negative bacteria (41). A 1.6-kb DNA fragment containing the *ant(4')-IIa* gene from *P. aeruginosa* was isolated (41). We determined the DNA sequence, developed a probe, and examined the microbial

distribution of the cloned *P. aeruginosa ant(4')-IIa* gene (90).

A 204-bp *ant(4')-IIa* probe was used to screen a collection of ANT(4')-I and ANT(4')-II strains from Europe and the United States (90). Most (83%) of the ANT(4')-II strains hybridized to the *ant(4')-IIa* probe, including most of the *Citrobacter freundii*, *Enterobacter cloacae*, *E. coli*, *Klebsiella* spp., *Morganella* spp., *Pseudomonas* spp., and *Serratia* spp. However, the presence of seven strains which expressed an ANT(4')-II profile but did not hybridize to either the *ant(4')-Ia* or *ant(4')-IIa* probe suggests the existence of a third *ant(4')* gene in some gram-negative bacteria (90).

ANT(6). (i) ANT(6)-I. The ANT(6)-I AGRP is characterized by resistance to streptomycin (68). This AGRP is found in gram-positive organisms. In a recent study, 80% of staphylococci and 87.6% of enterococci hybridized to the *ant(6)-Ia* probe (68). The remaining strains are likely to contain other streptomycin-modifying enzymes. Furthermore, of ~1,000 staphylococci and enterococci tested, 156 streptomycin-susceptible organisms hybridized to the *ant(6)-Ia* probe (68). The large number of false-positive strains suggests either the presence of cryptic chromosomal genes, such as observed with the *aac(6')-Ic* gene, or remnant pseudo-*ant(6)-Ia* genes in these strains.

ANT(9). (i) ANT(9)-I. This AGRP is characterized by resistance to spectinomycin only (20, 63). The gene encoding this enzyme was cloned from *Staphylococcus* transposon Tn*554* (63). This AGRP is unique to *Staphylococcus aureus* (63) and has not been observed in *Enterococcus* spp. (68). Although the resistance spectra of the ANT(9)-I and ANT(3")-I enzymes differ (spectinomycin versus spectinomycin plus streptomycin), the two proteins show 61% sequence similarity and 34% sequence identity, suggesting a common origin.

The arrangement of the *ant(9)-Ia* gene within Tn*554* was shown to be unusual. Murphy (63) found that the 3' end of the *ant(9)-Ia* transcript was located within 10 bp of the stop codon for the adjacent but convergently transcribed *ermA* gene (which encodes erythromycin resistance). A large inverted repeat separates the two genes, which probably functions as a rho-independent terminator for the *ant(9)-Ia* gene (63). The 3' ends of the two transcripts may overlap (63). This arrangement differs from what has been observed for other transposons, in which resistance genes have been shown to be arranged in a tandem (head-to-tail) order, and are likely to be transcribed from a common promoter (29, 66, 83, 93).

Phosphorylation

APH(3'). The APH(3') AGRPs are characterized by resistance to kanamycin and neomycin (62). At least seven unique APH(3') resistance mechanisms have been defined by extended resistance profiles to butirosin, lividomycin, amikacin, isepamicin, and gentamicin B (Table 1).

(i) APH(3')-I. The APH(3')-I AGRP is characterized by resistance to kanamycin, neomycin, paromomycin, ribostamycin, lividomycin, and gentamicin B (58). This AGRP is very commonly observed. However, the precise frequency varied among different species: 46% of gram-negative bacteria, 6.6% of *Pseudomonas* spp., 27.5% of *Serratia* spp., and 49.5% of *Acinetobacter* spp. (Table 1). Three genes which encode this AGRP have been cloned: *aph(3')-Ia* from Tn*903* (65); *aph(3')-Ib* from plasmid RP4 (69); and *aph(3')-Ic* from an isolate of *Klebsiella pneumoniae* which showed increased resistance to killing by neomycin (51). An *aph(3')-Ia* probe hybridized to most (90.5%) of the isolates which express kanamycin and neomycin resistance (Table 1). The *aph(3')-Ic* gene is nearly identical to *aph(3')-Ia* (seven nucleotide substitutions) (51), and therefore these strains would hybridize with the *aph(3')-Ia* probe. However, since the *aph(3')-Ia* and *aph(3')-Ib* genes share only 60% DNA homology, it is likely that the *aph(3')-Ia* probe would not hybridize with strains carrying the *aph(3')-Ib* gene under the stringent hybridization conditions used (89).

(ii) APH(3')-II. The APH(3')-II AGRP is characterized by resistance to kanamycin, neomycin, paromomycin, ribostamycin, butirosin, and gentamicin B (58). Thus, APH(3')-I and APH(3')-II differ in the ability to confer resistance to lividomycin and butirosin, respectively. A previous study has shown that although phosphorylation of amikacin can be detected in vitro, resistance to amikacin is not conferred, presumably because of the high K_m value (72). In that study, the combination of a chromosomal mutation which reduced the general uptake of aminoglycosides and a second mutation which increased the copy number of the plasmid carrying the *aph(3')-IIa* gene led to high level amikacin resistance (72).

A single gene which encodes the APH(3')-II AGRP has been identified, and this gene is associated only with transposon Tn*5* (3). This gene was rarely found in the clinical isolates studied—only 2.5% of the strains which were resistant to kanamycin and neomycin carried the *aph(3')-IIa* gene. The *aph(3')-IIa* gene is observed in gram-negative organisms and *Pseudomonas* spp. (Table 1).

(iii) APH(3')-III. The APH(3')-III AGRP is characterized by resistance to kanamycin, neomycin, paromomycin, ribostamycin, lividomycin, butirosin, and gentamicin B (16, 17). Amikacin and isepamicin are also modified in vitro, but many strains express only a low level of resistance when National Committee for Clinical Laboratory Standards susceptibility criteria are used (104). This AGRP is commonly found in gram-positive bacteria but has also been observed in *Campylobacter* spp. (49). The *aph(3')-IIIa* gene has been cloned and sequenced from *Staphylococcus aureus* (27) and *Streptococcus faecalis* (103).

(iv) APH(3')-IV. The APH(3')-IV AGRP is characterized by resistance to kanamycin, neomycin, paromomycin, ribostamycin, and butirosin. The *aph(3')-IVa* gene was cloned from a butirosin-producing strain of *Bacillus circulans* (35) and could be expressed in *E. coli* and *Streptomyces lividans*. Two potential overlapping promoters were identified upstream of the translational start: one resembled a vegetative promoter recognized by the σ^{55} form of RNA polymerase (now called σ^{43}), and the second was similar to developmentally regulated promoters recognized by σ^{37} (35). This arrangement is consistent with the hypothesis that a low constitutive level of APH(3')-IV protein is made during exponential growth and that high-level transcription from the σ^{37} promoter, as cells enter the stationary phase, could accompany the increase in antibiotic production (35).

The distribution of the APH(3')-IV AGRP and the *aph(3')-IVa* gene among other organisms has not been examined.

(v) APH(3')-V. The APH(3')-V AGRP is characterized by resistance to neomycin, ribostamycin, and paromomycin (39, 80). Three genes encoding this AGRP have been identified in streptomycetes: *aph(3')-Va*, cloned from a *Streptomyces fradiae* neomycin-producing strain (101); *aph(3')-Vb*, cloned from *Streptomyces ribosidificus* (ribostamycin-producing) (39); and *aph(3')-Vc*, cloned from a neomycin-producing strain of *Micromonospora chalcea* (80). The three

coding regions share strong DNA homology (80%), and this homology extended to 20 bp upstream of the transcriptional start but not into the −35 region (39, 80). Another conserved region was observed near the −60 region (80). Unlike *aph(3')-IVa* gene, only a single transcriptional start was identified in *aph(3')-Vc* from *M. chalcea* and *aph(3')-Vb* from *S. ribosidificus* (39, 80). The distribution of these genes in other organisms has not been examined.

(vi) APH(3')-VI. The APH(3')-VI AGRP is characterized by resistance to kanamycin, neomycin, paromomycin, ribostamycin, butirosin, and gentamicin B, as well as amikacin and isepamicin (48, 110). This AGRP differs from APH(3')-III by the lack of resistance to lividomycin (48). APH(3')-VI is associated primarily with *Acinetobacter* spp. (35.4%) and was rarely observed in total gram-negative bacteria (0.8%), *Pseudomonas* spp. (1.6%) and *Serratia* spp. (0.2%) (Table 1).

The *aph(3')-VIa* gene was cloned from *Acinetobacter baumannii* (57). A probe developed from this gene hybridized to most (82.7%) of the strains with this AGRP (Table 1). These data are consistent with the results of a previous study, in which 95% of amikacin-resistant *Acinetobacter* strains hybridized with the *aph(3')-VIa* probe (47). It is likely that an additional gene(s) encodes this resistance profile.

Another *aph(3')-VI* gene, showing the same resistance profile as APH(3')-VI, was cloned from *K. pneumoniae*/pRPG101 (25). DNA hybridization studies are necessary to show the relationship between this gene [*aph(3')-VIb*] and the *Acinetobacter baumannii aph(3')-VIa* gene.

(vii) APH(3')-VII. The APH(3')-VII AGRP is characterized by resistance to kanamycin and neomycin (95). In addition, phosphocellulose-binding assays showed that amikacin could be modified by this enzyme. The MICs of amikacin were ≤2.0 for 11 *Campylobacter jejuni* and 6 *Campylobacter coli* isolates, suggesting that amikacin resistance, as measured by National Committee for Clinical Laboratory Standards criteria, was not conferred (95). However, it is possible that a low level of amikacin resistance is conferred by the *aph(3')-VIIa* gene, similar to that observed in an APH(3')-III profile. To test whether these strains show an increase in amikacin MICs, a matched set of isolates (e.g., *E. coli* with and without the plasmid) must be compared.

The *aph(3')-VIIa* gene was cloned from *Campylobacter jejuni* (97). The % G+C ratio of the *aph(3')-VIIa* gene is consistent with the chromosomal content of *Campylobacter jejuni* (97). However, DNA hybridization studies are necessary to determine whether this gene is derived from a *Campylobacter* cellular gene and whether this plasmid-borne gene is now found in other bacteria.

APH(3"). **(i) APH(3")-I.** The *aph(3")-Ia* gene encodes a phosphotransferase specific for the 3"-hydroxy group of streptomycin (34). It was cloned from the streptomycin-producing strain *Streptomyces griseus* N2-3-11 (34). Unlike the gene which encodes the APH(6)-Ia enzyme, the *aph(3")-Ia* gene is not clustered with the enzymes involved in streptomycin production (56).

Two genes (*strA* and *strB*) which encode resistance to streptomycin were cloned from plasmid RSF1010 (84). Scholz et al. (84) reported that the first gene, *strA*, exhibited significant DNA homology with the kanamycin resistance-encoding gene, *aph(3')-IIa*, from Tn*5*. We find that these genes are 47% identical, whereas *aph(3")-Ia* and *strA* are 41% identical. However, our analysis of the protein sequence data indicates that the protein encoded by *strA* is significantly more related to the APH(3")-Ia protein (68% homology, 50% identity) than it is to the APH(3)-IIa protein (56% homology, 34% identity. These data, taken with the observation that both *strA* and *aph(3")-Ia* encode resistance theto streptomycin whereas *aph(3')-IIa* does not, suggested that the product of the *strA* gene is more likely to be an APH(3")-type enzyme, and therefore we have tentatively renamed the gene and protein *aph(3")-Ib* and APH(3")-Ib, respectively (Table 1). Final assignment to the specific class awaits further biochemical analysis.

APH(6). **(i) APH(6)-I.** Four additional genes encode streptomycin phosphotransferases which modify the 6-hydroxy group. The *aph(6)-Ia* gene was cloned from the streptomycin-producing strain *Streptomyces griseus* N2-3-11 and was found clustered with the enzymes involved in streptomycin production (22, 56). The equivalent gene [*aph(6)-Ib*] was cloned from *Streptomyces glaucescens*, a hydroxystreptomycin producer (36, 56, 108). The third gene, *aph(6)-Ic*, is encoded by the central region of Tn*5*. A polycistronic transcriptional unit includes *aph(3')-IIa* (kanamycin resistance), *ble* (bleomycin resistance), and *aph(6)-Ic* (streptomycin resistance) (60). The fourth gene, *strB*, was one of two genes encoding streptomycin resistance which were cloned from RSF1010 (84). On the basis of protein sequence homology with other members of the APH(6)-I family, especially the streptomycin resistance-encoding product of the *aph(6)-Ic* gene [APH(6)-Ic], *strB* has been tentatively renamed *aph(6)-Id* pending further biochemical analysis (Table 1).

APH(4). **(i) APH(4)-I.** The APH(4)-I AGRP encodes resistance to hygromycin B (28). Two APH(4)-I proteins have been identified: the protein encoded by the *aph(4)-Ia* gene, isolated from a strain of *E. coli* carrying pJR225; and a protein encoded by the *aph(4)-Ib* gene, isolated from the hygromycin B-producing strain *Streptomyces hygroscopicus* (28, 113). The *aph(4)-Ia* gene from *E. coli* was shown to reside downstream of but within the same transcriptional unit as *aac(3)-IVa* (19, 28). The protein sequences encoded by these two *aph(4)-I* genes are not closely related (Fig. 1).

Most of the probes summarized in Table 1 and in a previous study (89) hybridized with only 20 to 90% of the AGRP-positive strains. These data suggest that most AGRPs in gram-negative bacteria are due to more than one gene. The large numbers of genes which lead to similar resistance profiles had not previously been detected by classical techniques. In contrast, a recent study of 1,000 gram-positive cocci showed a remarkable homogeneity of genes when four different DNA probes from aminoglycoside-modifying enzymes were used (68). Probes from the *aph(3')-IIIa*, *aph(2")+aac(6')*, and *ant(4')-Ia* genes hybridized to ~100% of the strains containing the corresponding AGRP and 85% of the streptomycin-resistant strains hybridized with the *ant(6)-Ia* probe. These data emphasize that although there is a great diversity of aminoglycoside-modifying enzymes, most of these genes are currently restricted to gram-negative bacteria. This phenomenon may be due to different requirements for gene expression, plasmid replication, and barriers to genetic exchange.

STRUCTURE-FUNCTION RELATIONSHIPS AMONG FAMILIES OF AMINOGLYCOSIDE-MODIFYING ENZYMES

Previous reports have shown that there is significant protein sequence homology among some of the aminoglycoside-modifying enzymes, as well as homology to other proteins with related functions (1, 10, 57, 73, 81, 88, 103). A

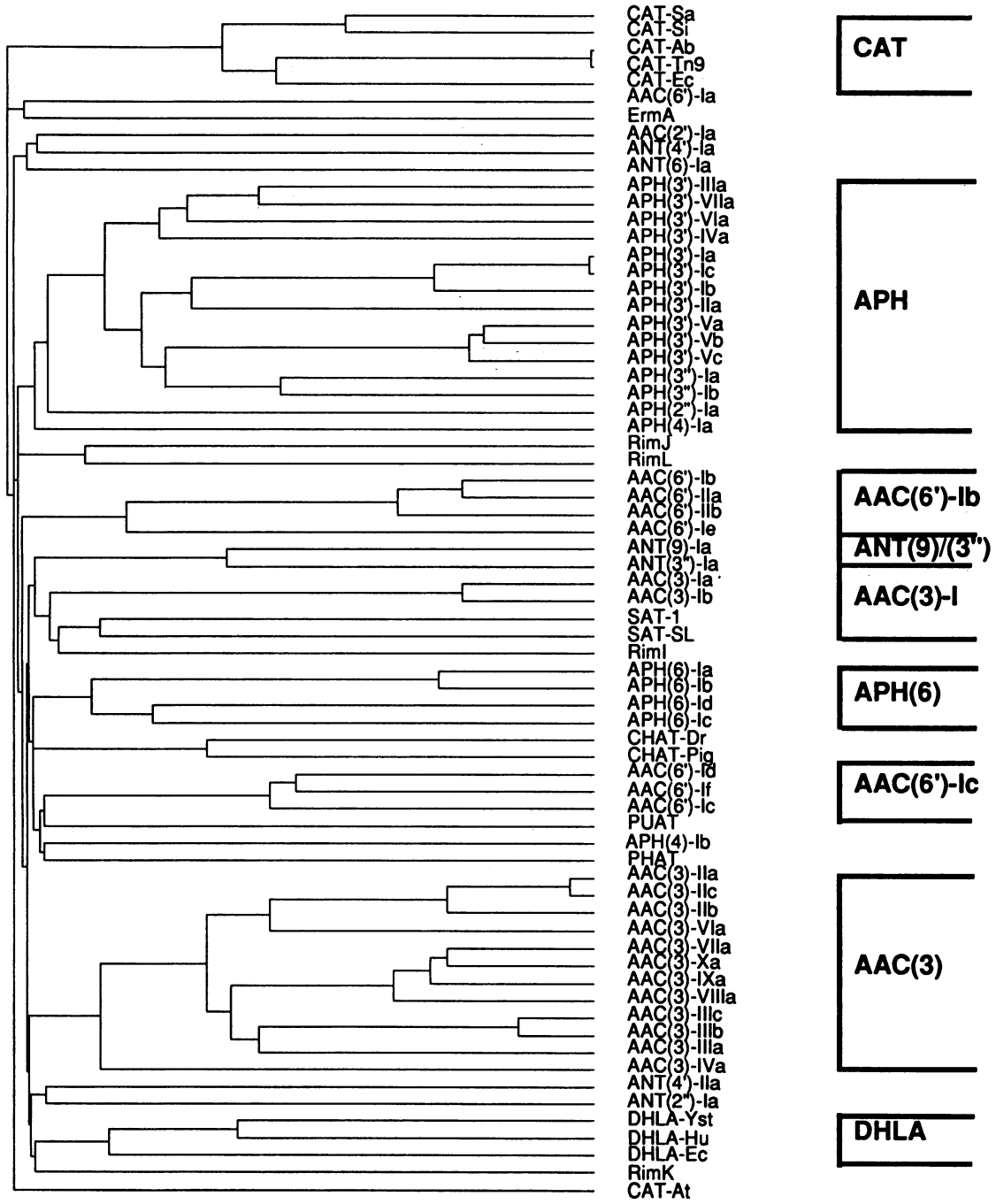

FIG. 1. Comparison of aminoglycoside-modifying enzymes and other acetyltransferases. Alignment of protein sequences was performed by the Pileup Multiple Sequence Analysis Program software package of the University of Wisconsin Genetics Computer Group (21). Abbreviations: CAT, chloramphenicol acetyltransferase; AAC, aminoglycoside acetyltransferase; Erm, erythromycin methyltransferase; APH, aminoglycoside phosphotransferase; Rim, acetylation of 30S ribosomal subunit; CHAT, choline acetyltransferase; ANT, aminoglycoside adenylyltransferase; DHLA, dihydrolipoamide acetyltransferase; SAT, streptothricin acetyltransferase.

complete analysis of all known aminoglycoside-modifying enzymes is shown in Fig. 1. Protein sequences predicted from all of the cloned aminoglycoside-modifying enzymes were compared by using the Pileup Multiple Sequence Analysis Program software package of the University of Wisconsin Genetics Computer Group (21). Several other known acetyltransferases were included in this analysis to test whether conserved sequences could be observed among enzymes which, for example, bind acetyl coenzyme A. In agreement with previous studies, the data in Fig. 1 show the clustering of similar sequences into families. Several distinct subfamilies could be identified: (i) APH, which included all of the known 3′-phosphorylating enzymes; (ii) AAC(6′)-Ib, AAC(6′)-IIa, AAC(6′)-IIb, and the AAC(6′) portion of the AAC(6′)+APH(2″) bifunctional protein; (iii) ANT(9) and ANT(3″), two enzymes which modify streptomycin; (iv)

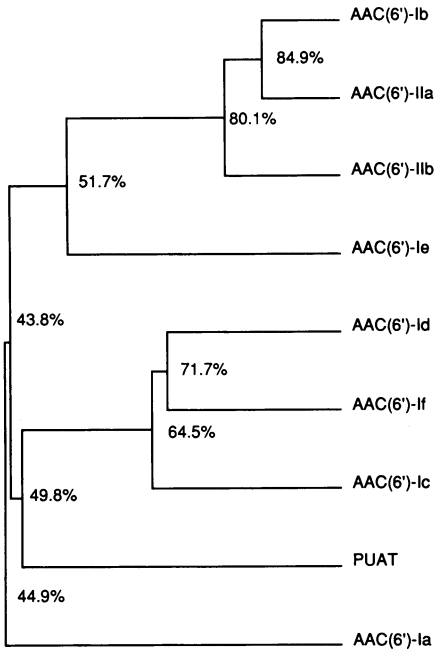

FIG. 2. Percent amino acid similarity in the AAC(6') family. Alignment of protein sequences was performed by the Pileup Multiple Sequence Analysis Program (see above) and displayed graphically. The percent similarity between two sequences is shown; the average percent similarity is shown where several sequences are interconnected. Values for amino acid similarity are taken from reference 85.

AAC(3)-Ia and AAC(3)-Ib; (v) APH(6)-I enzymes; (vi) AAC(6')-Ic, AAC(6')-Id, AAC(6')-If, and puromycin acetyltransferase (PUAT); and (vii) AAC(3) enzymes.

Several of the aminoglycoside-modifying enzymes did not fall into any distinct family (Fig. 1). These included AAC(6')-Ia, ANT(6)-Ia, ANT(4')-Ia, ANT(4')-IIa, APH(4)-Ib, ANT(2")-Ia, and AAC(2')-Ia. The chloramphenicol acetyltransferases, ErmA, and the dihydrolipoamide acetyltransferase (DHLA) enzymes made up separate subfamilies.

AAC(6') Family of Proteins

The AAC(6')-I and AAC(6')-II enzymes represent classes of bacterial proteins capable of acetylating tobramycin, netilmicin, and 2'-*N*-ethylnetilmicin. However, there is an important difference in their ability to modify amikacin and gentamicin (92). The AAC(6')-I enzymes modify amikacin and gentamicin C_{1a} and C_2, in contrast to the AAC(6')-II enzymes, which modify all gentamicin C compounds but not amikacin. At least nine different *aac(6')-I* genes have been identified, and the DNA sequence of at least six of the genes [*aac(6')-Ia* through *aac(6')-If*] encoding these enzymes have been determined (Table 1). In addition, two genes encoding AAC(6')-II enzymes have been cloned and sequenced (74a, 88). Analysis of the predicted amino acid sequence of eight of these proteins revealed that they are clustered within three distinct families (Fig. 1 and 2). However, we have identified common regions in these proteins, including two large motifs (Fig. 3). These regions may play important roles in the specificity of the enzyme for binding the aminoglycoside substrate and in catalysis (see below).

The largest AAC(6') subfamily is composed of AAC(6')-Ib, AAC(6')-IIa, AAC(6')-IIb, and the amino-terminal portion of the AAC(6')+APH(2") bifunctional enzyme [AAC (6')-Ie] (Fig. 2). Nucleotide sequence comparison of the *aac(6')-Ib* gene (64, 102) and the *aac(6')-IIa* gene showed 74% sequence identity (88). Comparison of the deduced protein sequences showed 76% identity and 85% amino acid similarity (88) (Fig. 2 to 4). The *aac(6')-IIb* gene was cloned from a *Pseudomonas* strain which contained an AAC(6')-II AGRP but did not hybridize to *aac(6')-Ib* (74a), which was used as a probe for the *aac(6')-IIa* gene (88, 89). DNA sequence analysis showed that although the *aac(6')-IIa* and *aac(6')-IIb* genes shared only 66% DNA sequence identity, the predicted protein sequences were 80% similar. A fourth member of this family is the amino-terminal portion of the AAC(6')+APH(2") bifunctional enzyme. This protein domain is more distantly related and shares 52% sequence similarity with the other three members of this family. The distribution of these enzymes is consistent with their relatedness: the AAC(6')+APH(2") bifunctional enzyme is restricted to gram-positive bacteria, whereas AAC(6')-Ib, AAC(6')-IIa, and AAC(6')-IIb have been observed only in gram-negative bacteria.

We have conducted a genetic analysis of the AAC(6')-Ib and AAC(6')-IIa enzymes to determine which amino acids were responsible for the differences in specificity (77). Results of domain exchanges, which created hybrid genes, indicated that amino acids in the carboxy half of the proteins determined the specificity. Mutations changing the specificity of the AAC(6')-Ib enzyme to that of the AAC(6')-IIa enzyme (i.e., gentamicin resistance and amikacin sensitivity) have been isolated. DNA sequence analysis of four independent isolates revealed base changes causing the same amino acid substitution, leucine to serine (motif 3, position 120) (Fig. 4). Interestingly, this serine occurs naturally at the same position in both AAC(6')-II enzymes (74a, 88). Oligonucleotide-directed mutagenesis was used to construct the corresponding amino acid change, serine to leucine, in the AAC(6')-IIa enzyme. This change resulted in the conversion of the AAC(6')-II substrate specificity to that of the AAC(6')-I enzyme (77). Results of the experiment on amino acid substitutions within this region and the conservation of this motif among the AAC(6')-I proteins (Fig. 3 and 4) suggest that we have located an aminoglycoside-binding site in this family of proteins (77). This is the first reported example of the putative identification of an aminoglycoside-binding site within these modifying enzymes.

Comparison of the protein sequences of AAC(6')-Ib, AAC(6')-IIa, AAC(6')-IIb, and AAC(6')-Ie reveals considerable sequence homology (Fig. 4). However, several regions which are conserved in AAC(6')-Ib, AAC(6')-IIa, and AAC(6')-IIb but not in AAC(6')-Ie are seen, including residues 43 to 46 (motif 1), 78 to 80, 99 to 102, and 105 to 108 (Fig. 4). One or all of these regions in the AAC(6')-Ie protein may encode the unique amino acid sequences necessary for binding and/or acetylation of fortimicin, which is observed only with the AAC(6')-Ie enzyme. Selection of fortimicin resistance mutants in strains carrying one of the *aac(6')-I* or *aac(6')-II* genes and analysis of the sequence alterations may reveal the amino acids sequences which are necessary for the additional modification of this aminoglycoside.

A second AAC(6')-I subfamily is composed of AAC(6')-Ic, AAC(6')-Id, and AAC(6')-If. The protein encoded by the *aac(6')-Ic* gene (91) was 47% identical and 62% similar to an AAC(6')-I protein from plasmid pU0490, isolated from *Enterobacter cloacae* (100), which we have designated AAC(6')-If (Table 1; Fig. 2). The AAC(6')-Ic protein was also 48% identical and 67% similar to the predicted protein

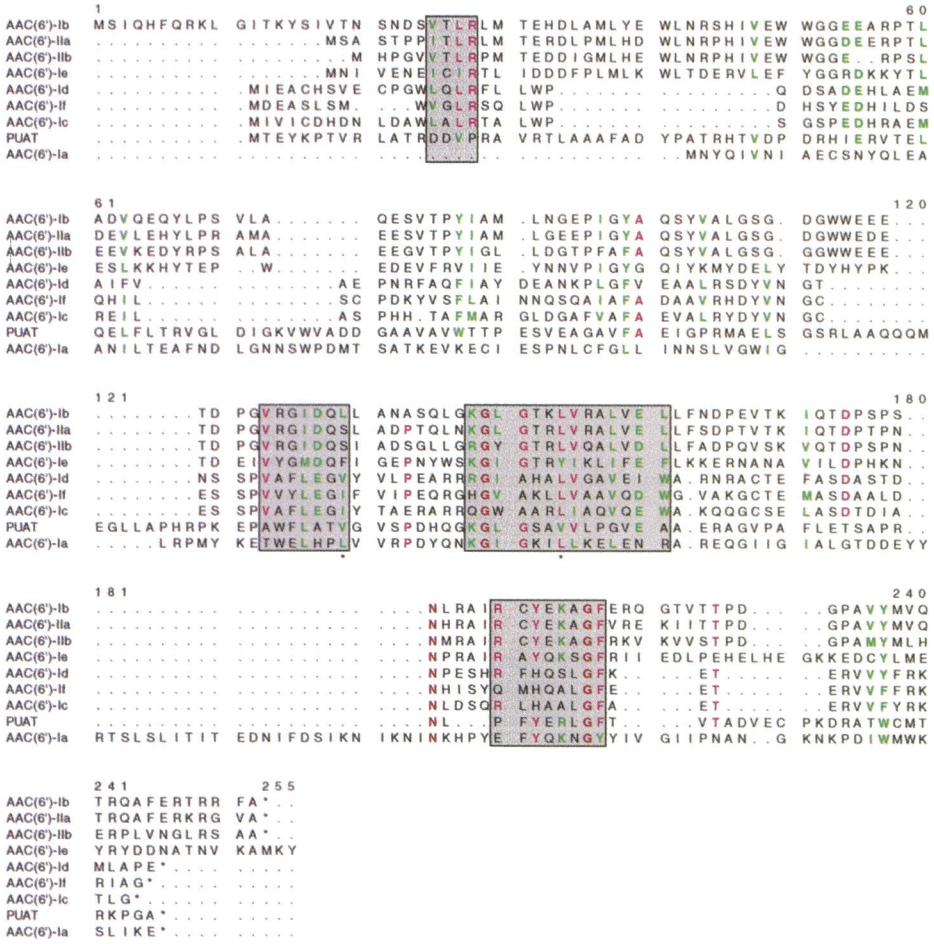

FIG. 3. Alignment of the AAC(6′) family of enzymes. Alignment of protein sequences was performed by the Pileup Multiple Sequence Analysis Program (see above). Key: red, all nine amino acids are identical; pink, at least six of the nine amino acids are identical; green, at least six of the nine amino acids are in one the similarity groups (C, Y), (D, E), (K, R), (F, L, W, Y), and (I, L, V, M).

encoded by an open reading frame from Tn*4000* located between the *ant(3″)-Ia* and *ant(2″)-Ia* genes (83, 91). Although it is not clear whether this open reading frame was expressed, the putative protein, which we have designated AAC(6′)-Id, is 72% similar to AAC(6′)-If (83, 100) (Fig. 2 and 5).

The high degree of relatedness of the AAC(6′)-Id and AAC(6′)-If proteins strongly suggested that they were recently derived from a common ancestral gene (Fig. 2 and 5). The gene encoding the more divergent chromosomally encoded AAC(6′)-Ic protein could be the common ancestor of both of these plasmid-borne genes. Alternatively, if the *aac(6′)-Id* and *aac(6′)-If* genes were derived from a non-*Serratia* bacterium, the *aac(6′)-Ic* gene could represent a *Serratia* gene which is homologous to the ancestral gene.

The third subfamily of AAC(6′)-I enzymes has only a single member, AAC(6′)-Ia. This protein is the least related of the AAC(6′) proteins but does show some sequence conservation, especially in the two largest motifs (Fig. 3). It had been previously reported that the AAC(6′)-Ia protein falls within a distantly related class of acetyltransferases which included AAC(3)-Ia, phosphinothricin acetyltransferase (PHAT), puromycin acetyltransferase (PUAT), and a ribosomal protein acetyltransferase (RimI) (73). According to the analysis in Fig. 1, AAC(6′)-Ia does not appear to be related to these proteins. RimI shows better alignment with the AAC(3)-I proteins, and PUAT appears to be unrelated to AAC(6′)-Ia. The PHAT and PUAT proteins do, however, show 46 and 50% sequence similarity, respectively, to the AAC(6′)-Ic, AAC(6′)-Id, AAC(6′)-If family of proteins (Fig. 1 and 2). PHAT, PUAT, and AAC(6′)-Ia are as related to the AAC(6′)-Ic and AAC(6′)-Ib families as they are to each other. These proteins do show sequence conservation within the motifs indicated (Fig. 3; data not shown). Examination of two additional acetyltransferase protein sequences, porcine choline acetyltransferase (6) and *Drosophila* choline acetyltransferase (94), revealed limited sequence similarity between these two related proteins and the AAC(6′)-Ic, AAC(6′)-Id, AAC(6′)-If family of enzymes, which clustered at the central region of the two larger motifs (87a). Conservation of these two regions across the three families of AAC(6′) proteins, as well as the choline acetyltransferase, PUAT, and PHAT enzymes, suggests that this region may be involved in a conserved active site. Interestingly, a leucine-to-serine substitution at the conserved Leu (position 154 within the largest motif) of the AAC(6′)-Ic protein (Fig. 3) leads to a specific loss in amikacin resistance, whereas the resistance to other aminoglycosides remains the same (78a). These data are consistent with the idea that this region is involved with the size or shape, or both, of the active site.

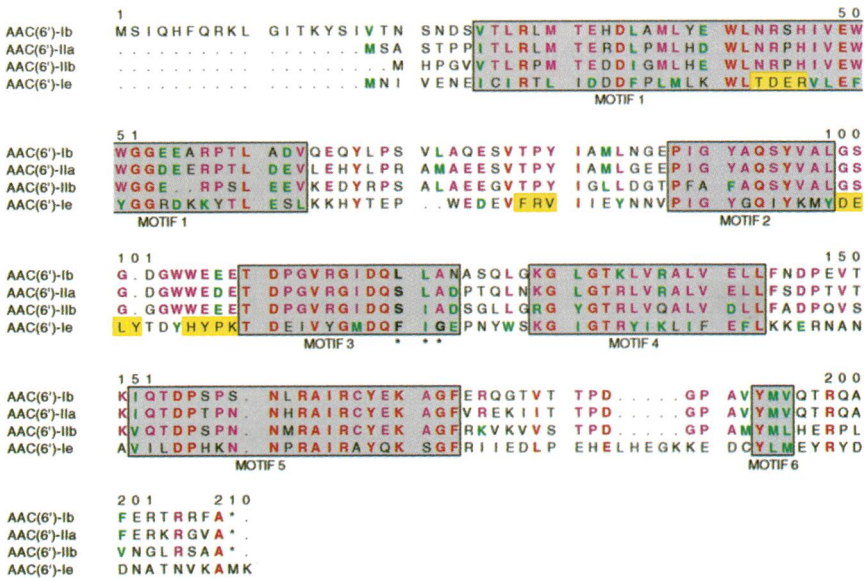

FIG. 4. Sequence similarity among the AAC(6')-IIa, AAC(6')-IIb, AAC(6')-Ib, and AAC(6')-Ie enzymes. Alignment of protein sequences was performed by the Pileup Multiple Sequence Analysis Program (see above). Key: red, all four amino acids are identical; pink, at least three of the four amino acids are identical; green, at least three of the four amino acids are in one the similarity groups (C, Y), (D, E), (K, R), (F, L, W, Y), and (I, L, V, M); yellow, sites possibly involved in resistance to fortimicin.

Additional members of the AAC(6') family of proteins will probably be found. DNA hybridization analysis of 3,842 gram-negative bacteria, including 460 strains which showed an AAC(6') profile but did not hybridize to *aac(6')-Ia*, *aac(6')-Ib*, or *aac(6')-Ic* probes, suggested that other genes may be responsible for the AAC(6')-I resistance profile observed in these strains. Another two *aac(6')-I* genes [*aac(6')-Ig* and *aac(6')-Ih*] have been cloned from *Acinetobacter* strains and encode proteins which are members of the AAC(6')-Ic, AAC(6')-Id, AAC(6')-If family of enzymes (46a). The *aac(6')-Ii* gene has been cloned from an *Enterococcus faecium* strain (15).

AAC(3) Family of Proteins

Previous studies examined the sequences of five of the AAC(3) proteins and demonstrated that they showed significant homology (81). However, the AAC(3)-Ia protein was reported to lack homology with other members of the AAC(3) family and was defined as a member of a separate class of proteins (10, 81). We have cloned and characterized an additional five genes encoding members of the AAC(3) family of proteins: *aac(3)-Ib* (86), *aac(3)-IIb* previously designated *aac(3)-Vb* (77), *aac(3)-IIIb* (91a), *aac(3)-IIIc* (50), and *aac(3)-VIa* (75). Using the protein sequence data predicted from all of these genes and *aac(3)-IIc* (105), *aac(3)-IIIa* (107), and *aac(3)-Xa* (40), we have extended the original findings of protein homology and observed remarkable amino acid conservation in this family (Fig. 6 and 7).

A highly related cluster of AAC(3) enzymes is enzymes by genes cloned from actinomycetes [*aac(3)-VIIa*, *aac(3)-VIIIa*, *aac(3)-IXa*, *aac(3)-Xa*] (Fig. 6). The AAC(3)-VIIa enzyme has been reported to have an AGRP similar to that of AAC(3)-II (54). However, since the full aminoglycoside resistance spectrum encoded by each of these genes has not been reported, it is impossible to correlate amino acid changes with alterations in phenotype.

A second cluster of similar proteins includes the three AAC(3)-II enzymes and the more distantly related AAC(3)-VIa enzyme (Fig. 6). The AAC(3)-IIa and AAC(3)-IIc (105) proteins are nearly identical, showing differences in only 12 amino acids. Likewise, AAC(3)-IIb (77) is highly conserved

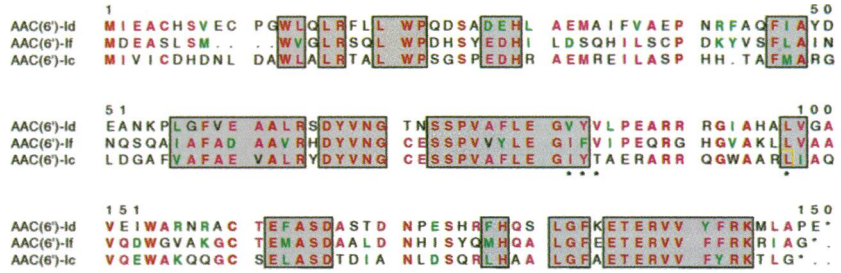

FIG. 5. Sequence alignment of the AAC(6')-Ic, AAC(6')-Id, and AAC(6')-If proteins. Alignment of protein sequences was performed by the Pileup Multiple Sequence Analysis Program (see above). Key: red, all three amino acids are identical; pink, at least two of the three amino acids are identical; green, at least two of the three amino acids are in one the similarity groups (C, Y), (D, E), (K, R), (F, L, W, Y), and (I, L, V, M).

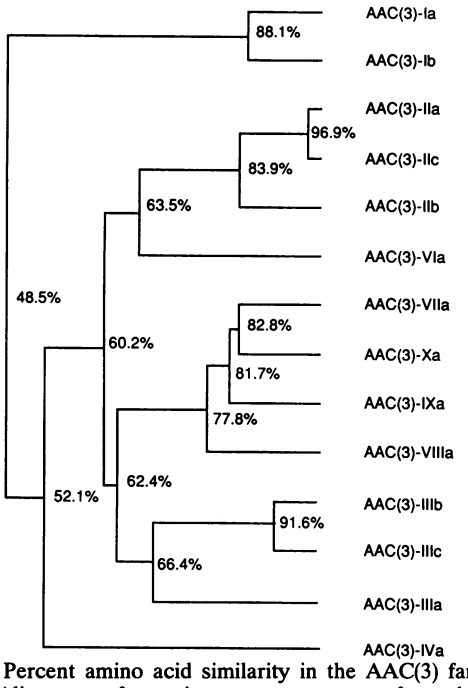

FIG. 6. Percent amino acid similarity in the AAC(3) family of enzymes. Alignment of protein sequences was performed by the Pileup Multiple Sequence Analysis Program (see above) and displayed graphically. The percent similarity between two sequences is shown; the average percent similarity is shown where several sequences are interconnected. Values for amino acid similarity are taken from reference 85.

and the amino acid sequence is 84% similar to both AAC(3)-IIa and AAC(3)-IIc (Fig. 6). Organisms expressing any of these three proteins have the same aminoglycoside resistance profile (gentamicin, tobramycin, netilmicin, 6′-*N*-ethylnetilmicin, and 2′-*N*-ethylnetilmicin) (78a). The gene encoding AAC(3)-VIa (75) is also a member of this cluster, although it shares less sequence similarity (63.5%) (Fig. 6). The AAC(3)-VI resistance profile was carefully determined by comparing the aminoglycoside MICs for the cloned gene and the susceptible, untransformed *E. coli*. The resistance profile is characterized by high resistance (≥32 μg/ml) to gentamicin and 6′-*N*-ethylnetilmicin, intermediate resistance (8 to 16 μg/ml) to tobramycin and netilmicin, and slightly elevated MICs (2 to 4 μg/ml) to 2′-*N*-ethylnetilmicin, suggesting that the ability to acetylate tobramycin, netilmicin, and 2′-*N*-ethylnetilmicin, which was seen with the AAC(3)-II enzymes, has decreased in the AAC(3)-VIa enzyme. These data are consistent with the enzymatic activity observed against each of the aminoglycoside substrates by using the phosphocellulose-binding assay (70). Comparison of the sequences of all of the AAC(3) enzymes reveals a single-amino-acid change at position 74, within a highly conserved motif, that could partially be responsible for this loss of function (Fig. 7). All of the AAC(3) enzymes encoding resistance to tobramycin have a conserved T (threonine) at this residue, whereas the three proteins which do not encode tobramycin resistance, AAC(3)-VIa, AAC(3)-Ia, and AAC(3)-Ib, have other, nonrelated amino acid substitutions at this position (N [asparagine], R [arginine], and K [lysine], respectively) (Fig. 7). Oligonucleotide-directed mutagenesis of this residue may reveal interesting structure-function information about the AAC(3) enzymes.

A third cluster consists of the three AAC(3)-III enzymes, whose corresponding genes have all been cloned from *Pseudomonas* spp. These enzymes show identical resistance profiles: gentamicin, tobramycin, and 5-episisomicin. The AAC(3)-IIIc protein is encoded by a gene which cross-hybridizes to an *aac(3)-IIIb* probe. The *aac(3)-IIIb* and *aac(3)-IIIc* genes are 83.6% identical, and the proteins encoded by these genes are 97% similar. DNA probes from *aac(3)-IIIb* or *aac(3)-IIIc* do not hybridize with *aac(3)-IIIa* from *P. aeruginosa* Travers under stringent conditions (87a). Consistent with this finding is that the AAC(3)-IIIa protein is only 66.4% similar to the AAC(3)-IIIb and AAC(3)-IIIc proteins. Unlike the AAC(3)-II enzymes, the AAC(3)-III enzymes do not acetylate netilmicin, 6′-*N*-ethylnetilmicin, or 2′-*N*-ethylnetilmicin. This "loss of function" could be due to a sequence alteration at position 149 (H [histidine]) (Fig. 7). Interestingly, AAC(3)-Ia and AAC(3)-Ib, which also do not acetylate the netilmicins, are altered at position 149 as well. AAC(3)-VIa, which also fails to acetylate the netilmicins, shows a serine-to-threonine substitution nearby at position 147, which may prevent interaction at the conserved histidine 149 (Fig. 7).

The AAC(3)-Ia and AAC(3)-Ib proteins share 88% amino acid similarity. These two proteins make up a separate AAC(3) cluster, only weakly related (48.5% similarity) to the other AAC(3) proteins. Piepersberg et al. (73) reported some sequence homology of AAC(3)-Ia, AAC(6′)-Ia, and RimI, which acetylates the N-terminal alanine of the S18, 30S ribosomal protein. The AAC(3)-I proteins are about equally related to the AAC(3) family of enzymes as they are to RimI and to two other streptothricin acetyltransferase proteins, SAT-I (33) and SAT-SL (38). The RimI/AAC(3)-I and SAT/AAC(3)-I regions of homology are localized to the middle and C-terminal ends of the proteins, whereas the RimI/AAC(6′)-Ia homology, which is much weaker, is limited to the central portion. There are only 24 amino acid residues which are similar in all five proteins, and these are localized predominantly to the central portion of the proteins. It is possible that the regions common among RimI, SAT-1, and the AAC(3)-I proteins involve the binding of acetyl coenzyme A or the active site.

AAC(2′) Protein

A chromosomal gene encoding AAC(2′)-Ia has been recently cloned from a *Providencia stuartii* strain (78). DNA sequence analysis revealed no homology to any known sequence. The single open reading frame encodes a protein of 179 amino acids. The hydrophobicity plot of the putative AAC(2′)-Ia protein was similar to that of the AAC(6′)-Ic protein (78), although there is no amino acid homology. This may reflect similarities in protein structure which may have evolved as a result of convergent evolution.

Phosphorylating Family of Proteins

The aminoglycoside phosphotransferase family of proteins has been previously shown to contain several conserved functional domains (57, 103). Three motifs, located toward the carboxy half of the protein, have been hypothesized to play specific functional roles (Fig. 8 and 9). Motif 1, V--HGD----N, may be involved in the catalytic transfer of the terminal phosphate upon ATP catalysis. This motif was detected in a variety of proteins which encode ATPase activity (57). Specifically, the histidine residue may be the phosphate-accepting residue in the phosphotransferase reac-

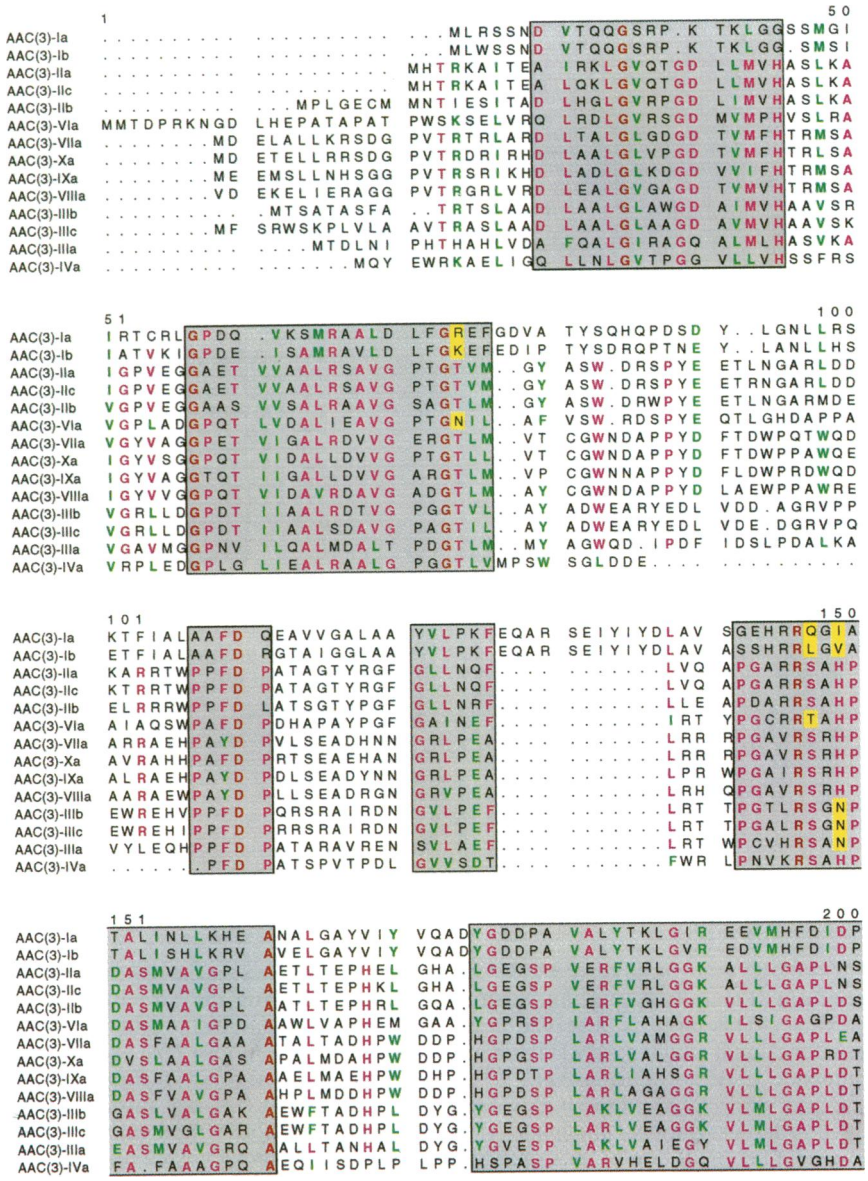

FIG. 7. Alignment of the AAC(3) family of enzymes. Alignment of protein sequences was performed by the Pileup Multiple Sequence Analysis Program (see above). Key: red, all 14 amino acids are identical; pink, at least 9 of the 14 amino acids are identical; green, at least 9 of the 14 amino acids are in one the similarity groups (C, Y), (D, E), (K, R), (F, L, W, Y), and (I, L, V, M); yellow, sites possibly important in the determination of the resistance profile.

tion (8, 57). Tyrosine and serine can also accept phosphate. Substitution of Tyr, Ser, or Leu for the His-188 residue (Fig. 9, position 239) or substitution of Asp for Gly-189 (Fig. 9, position 240) in the *aph(3')-IIa* gene results in a nonfunctional enzyme (kanamycin-susceptible phenotype) (8). Similarly, substitution of Gly for the conserved Asp-190 residue (Fig. 9, position 241) severity reduces the specific activity of the mutant enzyme and the level of resistance conferred (46). The importance of this motif to the function of the APH class of enzymes is clearly demonstrated by the two invariant residues (histidine at position 239 and aspartate at position 241) within this motif (Fig. 9).

Motif 2, G--D-GR-G may correspond to the "glycine-rich flexible loop" previously described as part of the nucleotide-binding site in several GTP- and ATP-binding proteins (57). A subset of this sequence binds Mg^{2+}-ATP to form a ternary complex composed of enzyme-ATP-Mg^{2+}. Blázquez et al. (8) showed that substitution of the conserved arginine-211 residue (Fig. 9, position 271) with glycine in the *aph(3')-IIa* gene resulted in a nonfunctional enzyme. In a similar study, several mutants were isolated with alterations in conserved amino acids within motif 2 (46). Asp-208 to Gly (Fig. 9, position 268), Gly-210 to Asp (position 270), Arg-211 to Gln (position 271), Asp-216 to Gly (position 276), and Asp-220 to Gly (position 281) all resulted in less than 6% of the wild-type level of kanamycin phosphorylation and a similar reduction in the level of aminoglycoside resistance conferred. On the other hand, a mutation outside of this conserved motif, Asp-227 to Gly (Fig. 9, position 288), was much less severe and retained 64% of the enzyme activity.

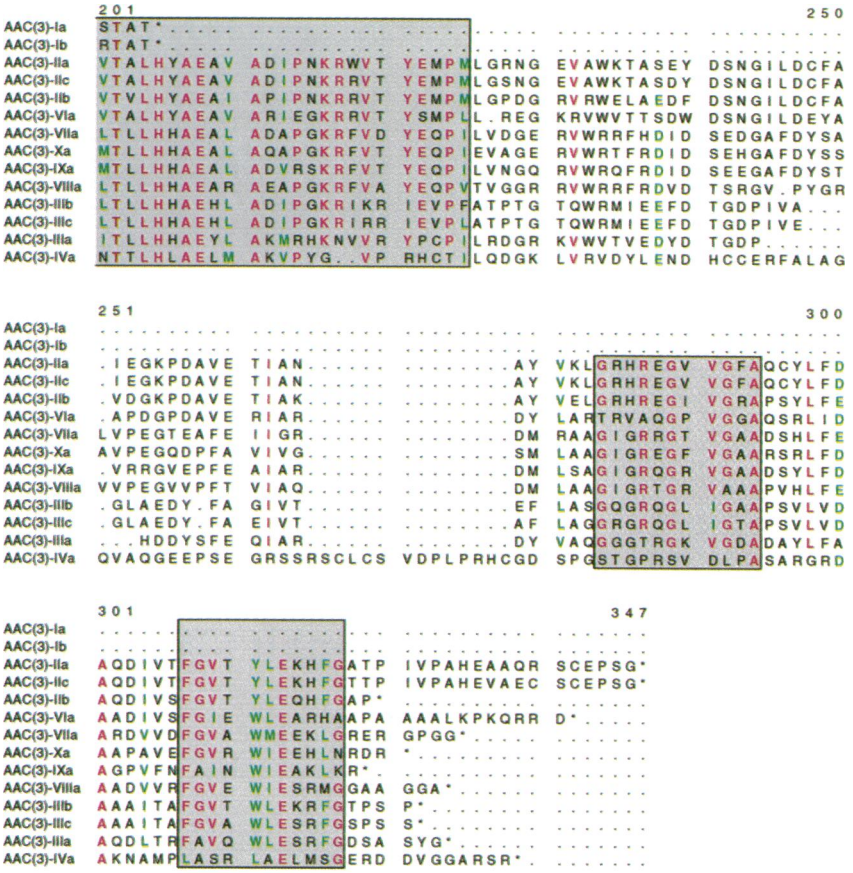

FIG. 7—*Continued.*

Motif 3, D--R/K--F/Y---LDE, may be involved ATP hydrolysis and/or in a conformational change in the enzyme-aminoglycoside complex (57). This motif is observed in the APH(2″) domain of the bifunctional enzyme, ANT(4′)-Ia and ANT(6)-Ia, and the sequence LDE is found in ANT(2″)-Ia, ANT(6)-Ia, ANT(3″)-Ia, and viral DNA-interacting proteins but not in aminoglycoside-acetylating enzymes (57). Blázquez et al. (8) showed that substitution of the conserved Asp-261 residue (Fig. 9, position 352) with Asn in the *aph(3′)-IIa* gene resulted in a nonfunctional enzyme. Furthermore, deletion of the DNA encoding the last 24 amino acids of the APH(3′)-IIa protein resulted in the loss of kanamycin resistance (3). These data suggest that motif 3 is essential for the function of these enzymes (Fig. 9).

The relatedness of the APH family of enzymes is shown in Fig. 8. In general, the members of the APH family are more dissimilar than the members of the AAC(3) family, which may be related to the larger diversity of phenotypes within the APH family. Because of the greater diversity, it is difficult to suggest specific amino acid changes which may be responsible for the observed alterations in the resistance spectrum.

APH(3′)-I. Two APH(3′)-I enzymes, APH(3′)-Ia and APH(3′)-Ib, which showed identical resistance profiles were 82.5% similar. A gene, which was closely related to *aph(3′)-Ia*, was cloned from a *Klebsiella pneumoniae* strain [*aph(3′)-Ic*, previously designated *aphA*1-IAB] (51). *E. coli* K-12 transformed with pBWH77, which carries the *aph(3′)-Ic* gene, showed greater resistance to killing by neomycin than did a control strain carrying the *aph(3′)-Ia* gene (51). The DNA sequence of the *aph(3′)-Ic* gene was nearly identical to *aph(3′)-Ia*, and the proteins differed by only 4 amino acid substitutions, which occurred within the first 80 amino acids of this protein (51) (Fig. 8 and 9). These changes were in nonconserved regions (Fig. 9, positions 27, 35, 57, and 94).

APH(3′)-II. Several missense mutations within the three conserved motifs of the *aph(3′)-IIa* gene which eliminated or reduced aminoglycoside resistance have been isolated (8, 44, 112) (Fig. 9). However, changing Tyr-218 to Ser or to Asp (Fig. 9, position 279) leads to an alteration in the substrate specificity of the enzyme, such that resistance to amikacin is increased eightfold and twofold, respectively. These alterations were associated with a concomitant decrease in the K_m for amikacin (44). Mutation from Tyr-218 to Phe (Fig. 9, position 279) did not show these effects but, rather, decreased resistance and increased the K_m for all of the aminoglycosides tested, except amikacin (44). Since Tyr-218 is within the conserved motif 2, which shares many features of a nucleotide-binding site in several GTP- and ATP-binding proteins, it is surprising that none of the mutations lead to alterations in the binding of Mg^{2+}-ATP but, rather, lead to an alteration in aminoglycoside specificity (44). It is possible that these mutations lead to an alteration in the substrate-binding site by affecting the accessibility or orientation of the reactive groups within the active site (45).

Other mutations have been isolated outside of the three previously identified motifs. Blázquez et al. (8) showed that conversion of Val-36 to Met (Fig. 9, position 58) resulted in a 20-fold decrease in the level of assistance. Although this region is not within motif 1, 2, or 3 it is likely that it is

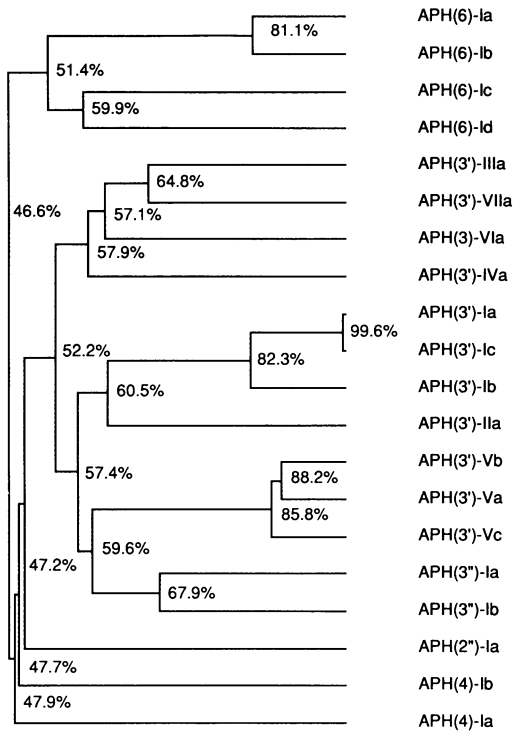

FIG. 8. Percent amino acid similarity in the APH family. Alignment of protein sequences was performed by the Pileup Multiple Sequence Analysis Program (see above) and displayed graphically. The percent similarity between two sequences is shown; the average percent similarity is shown where several sequences are interconnected. Values for amino acid similarity are taken from reference 85.

important to the function of the APH proteins since the conserved group of amino acids Ile, Leu, and Val are observed at this position in 18 of the 20 APH proteins examined.

A second mutation outside of the conserved regions, Glu-182 to Asp (Fig. 9, position 233), was also described (112). Although the mutant was resistant to kanamycin at 200 μg/ml, growth was retarded compared with that of the wild type in the presence of kanamycin at 400 μg/ml. This effect was even more pronounced with G418, in which the wild type grew normally at 30 μg/ml while no growth was observed in strains carrying the mutant enzyme.

APH(3')-V. Three *aph(3')-V* genes have been cloned from actinomycetes: *aph(3')-Va*, from a *Streptomyces fradiae* neomycin-producing strain (101); *aph(3')-Vb*, from *Streptomyces ribosidificus* (ribostamycin producing) (39); and *aph(3')-Vc*, from a neomycin-producing strain of *Micromonospora chalcea* (80). The proteins encoded by these genes are highly conserved (Fig. 8).

APH(3")-I and APH(6)-I. The APH(3")-I and APH(6)-I enzymes confer resistance to streptomycin. However, the APH(3")-Ia enzyme has been shown to modify streptomycin at the 3" position rather than at the 6-hydroxy group (56).

The APH(6)-I enzymes share only limited protein sequence homology (46.6%) with the APH(3') class of enzymes (Fig. 8). The gene which encodes the APH(6)-Ia enzyme was cloned from the same *Streptomyces griseus* strain as the *aph(3")-Ia* gene and was found clustered with the genes encoding the enzymes involved in streptomycin production (56). It is likely that the APH(6)-Ia protein is involved in streptomycin metabolism. Consistent with this theory is the fact that the APH(6)-Ia enzyme is more strongly expressed in the streptomycin production phase and that expression of the *aph(3")-Ia* gene has not been demonstrated in this strain (56).

The APH(6)-Ib protein from the hydroxystreptomycin-producing *Streptomyces glaucescens* is 81% similar and 73% identical to the APH(6)-Ia protein, and the *aph(6)-Ia* and *aph(6)-Ib* genes are 75% identical (56, 108). The high degree of relatedness of these two genes and the corresponding proteins is strong evidence for a common origin. Furthermore, Hintermann et al. (36) have shown that streptomycin-susceptible strains of *Streptomyces glaucescens*, which lack the ability to produce hydroxystreptomycin, contain large deletions of the *aph(6')-Ib* gene. If this gene is clustered with the genes involved in aminoglycoside production, as is the homologous *aph(6')-Ia* gene, it is possible that all or part of the hydroxystreptomycin biosynthesis cluster is deleted as well.

The APH(6)-Ic protein, encoded by Tn*5* (60), is 55% similar to both the APH(6)-Ia and APH(6)-Ib proteins. The APH(6)-Id protein, from plasmid RSF1010, is the most distantly related of the four APH(6) enzymes encoding streptomycin resistance, being 47% similar to APH(6)-Ia and 49% similar to APH(6)-Ib.

Unlike the APH(6)-I proteins, the APH(3")-I proteins share considerable amino acid homology with the APH(3') class of enzymes (34) (Fig. 8 and 9). Since the *aph(3")-Ia* gene was not genetically linked to the genes involved in streptomycin metabolism, it is likely that it did not derive from genes involved in streptomycin production (56).

APH(4)-I. Resistance to hygromycin B is mediated by the APH(4)-Ia and APH(4)-Ib proteins. Overall, these proteins are only 52% similar and 18% identical, but several regions show higher degrees of conservation. These regions correspond to the conserved motifs around positions 90 to 110, as well as the extended motifs 1 and 2 (Fig. 9). The data suggest that these proteins are not closely related and may share only some amino acid sequences common to all of the APH proteins (Fig. 8 and 9). A fourth conserved region showing 65% similarity and 27% identity was observed between amino acids 6 to 33 of the APH(4)-Ia protein and amino acids 1 to 26 of the APH(4)-Ib protein (data not shown). The significance of this conserved region is unknown.

Adenylylating Family of Proteins

ANT(2")-I. The DNA sequence of a single *ant(2")-Ia* gene has been determined (12, 37). The DNA sequence of a putative second *ant(2')-Ib* gene suggested that it had been misclassified (52). Phosphocellulose-binding assays have shown that this sequence actually encodes an AAC(3)-III enzyme (26a) (see above). The ANT(2")-Ia and AAC(3)-III proteins show no sequence conservation, nor does ANT(2")-Ia show significant homology to any other class of aminoglycoside-modifying enzyme (Fig. 1).

ANT(3")-I and ANT(9)-I. The *ant(3")-Ia* gene encodes resistance to streptomycin and spectinomycin, whereas the *ant(9)-Ia* gene encodes resistance to spectinomycin only. Although the resistance spectra of the enzymes encoded by these genes differ, the two proteins were 61% similar, suggesting a common origin (Fig. 1). There is no similarity between these two enzymes and other aminoglycoside-modifying enzymes.

ANT(4')-I and ANT(4')-II. The DNA sequence of the

ant(4')-IIa gene predicted a 262-amino-acid protein of 28.9 kDa (91a), whereas the *ant(4')-Ia* gene encodes a protein of 257 amino acids (59). Overall, the protein sequences were only 45% similar. However, additional analysis revealed that the ANT(4')-IIa and ANT(4')-Ia proteins show some sequence conservation near the amino-terminal end [54% similar for amino acids 2 to 87 of ANT(4')-IIa and amino acids 15 to 99 of ANT(4')-Ia]. This region could represent a common domain, such as an aminoglycoside- or ATP-binding site. However, it is not clear whether the similarity is due to divergent or convergent evolution. The ANT(4')-IIa and ANT(4')-Ia proteins have been previously shown to differ in the spectrum of aminoglycoside modification [e.g., ANT(4')-IIa cannot modify aminoglycosides at the 4"-hydroxyl position] (82, 87). There is no similarity between these enzymes and any of the other classes of enzymes.

ANT(6)-I. A single gene encoding ANT(6)-Ia has been cloned (67). The protein encoded by this gene shows no homology with any of the other aminoglycoside-modifying enzymes (Fig. 1).

ORIGIN AND MECHANISMS OF DISSEMINATION OF THE GENES ENCODING AMINOGLYCOSIDE-MODIFYING ENZYMES

It has long been speculated that the aminoglycoside resistance genes in clinically relevant strains were derived from organisms producing the aminoglycosides (4). The presence of these enzymes in aminoglycoside-producing strains could provide a mechanism of self-protection against the antibiotic produced. Therefore, the actinomycetes could have provided the initial gene pool from which some of the present-day aminoglycoside resistance genes were derived. Several genes encoding aminoglycoside-modifying enzymes, including *aph(3')-Va*, *aph(3')-Vb*, *aph(3')-Vc*, *aac(3')-VIIa*, *aac(3)-VIIIa*, *aac(3)-IXa*, and *aac(3)-Xa*, have been cloned from aminoglycoside-producing organisms. Others, such as *aph(6)-Ia*, have been shown to be genetically linked to the genes encoding enzymes involved in aminoglycoside production (Table 1).

A second theory is that aminoglycoside resistance genes are derived from bacterial genes which encode enzymes involved in normal cellular metabolism (73). According to this theory, the selective pressure of aminoglycoside usage causes mutations which alter the expression of these enzymes, resulting in the ability to modify aminoglycosides. The *aac(6')-Ic* gene of *S. marcescens* is an example of how aminoglycoside resistance can derive from modification of the regulation of a metabolic gene. We have determined the complete nucleotide sequence of this gene (91). DNA hybridization has shown that all *S. marcescens* strains have this gene, regardless of resistance profile, and primer extension analysis has shown that the *aac(6')-Ic* gene is transcriptionally silent in aminoglycoside-susceptible strains (91). Studies of the function of these genes in bacteria will shed light on the mechanisms which convert genes involved in cellular metabolism into aminoglycoside resistance genes.

Many of the genes encoding aminoglycoside-modifying enzymes are associated with transposable genetic elements. The DNA sequences of a large number of resistance genes, including those causing resistance to sulfonamides, mercuric ions, and streptomycin, have Tn*21* DNA flanking these genes (37, 66, 93). More recent data have suggested that Tn*21* contains a specific region, the integron, into which many different resistance genes have inserted (66). The integron consists of two conserved 59-bp elements flanking one or more inserted genes (29). Although the integron is most often found associated with Tn*21*, there are a few examples of integron sequences found independently (29, 66, 88). New resistance genes have been shown to reside within the integron in place of the *ant(3")-Ia* gene or inserted either 5' or 3' to the *ant(3")-Ia* gene. Up to three resistance genes have been shown to be arranged in tandem within the integron (29, 66, 93). The model invoked to explain the large numbers of different resistance genes present within the integron is that sequences within the repeated 59-bp elements serve as site-specific recombinational hot spots (66, 93). The sequence **GTT**, at the 3' end of the 59-bp element, is the crossover point for the insertion of new resistance genes into the integron (29). The genes are all inserted in the same orientation and are transcribed from a promoter within the 5'-conserved element. The presence of a strong promoter upstream of the insertional hot spot may ensure high-level expression of the inserted genes.

Several laboratories have described the association of genes encoding aminoglycoside-modifying enzymes with integron sequences, including *ant(2")-Ia* (12), *aac(3)-Ia* (98), *ant(3")-Ia* (37), and *aac(6')-Ia* (96). We have characterized two genes, *aac(6')-IIa* (88) and *aac(3)-VIa* (75), which are also present within an integron environment. DNA sequence analysis has shown that the *aac(6')-IIa* gene has inserted in place of the *ant(3")-Ia* gene (88). The *aac(3)-VIa* gene has inserted 3' to the *ant(3")-Ia* gene but does not appear to have inserted into the recombinational hot spot (75). Primer extension analysis has localized the *aac(3)-VIa* promoter to within the integron (74b). This promoter is active in at least two different bacterial hosts, providing further evidence for the selective advantage of insertions into the integron.

The ability of resistance genes to move to and from various replicons, some with a very broad host range, has allowed the rapid dissemination of these genes within bacteria. Aminoglycoside resistance surveys on strains isolated before 1983 showed that most strains contained only a single resistance mechanism (61, 74, 92). However, more recent surveys have demonstrated that most resistant strains now carry combinations of several aminoglycoside resistance mechanisms (30, 60a, 89). We have recently documented the existence of strains carrying up to six aminoglycoside resistance mechanisms (89). The discovery of multiple resistance genes within a single integron is one explanation for the emergence of multiply resistant strains. These findings have serious implications for the spread of these resistance genes within bacterial species.

REGULATION OF AMINOGLYCOSIDE RESISTANCE GENES

The aminoglycoside resistance genes, in general, do not appear to be regulated. Transcription of these genes is apparently constitutive and, although costly in terms of cellular energy, provides constant protection against the presence of aminoglycosides. However, two exceptions to this generalization are that the expression of the chromosomal *aac(6')-Ic* gene of *S. marcescens* (74b) and the *aac(2')-Ia* gene of *Providencia stuartii* appears to be tightly regulated (78).

Previous studies have shown that exposure of netilmicin-susceptible *S. marcescens* to increasing subinhibitory concentrations of netilmicin is associated with the appearance of an AAC(6')-I aminoglycoside resistance profile (31). Starting with eight strains that were aminoglyoside susceptible or that expressed resistance to only 2'-*N*-ethylnetilmicin

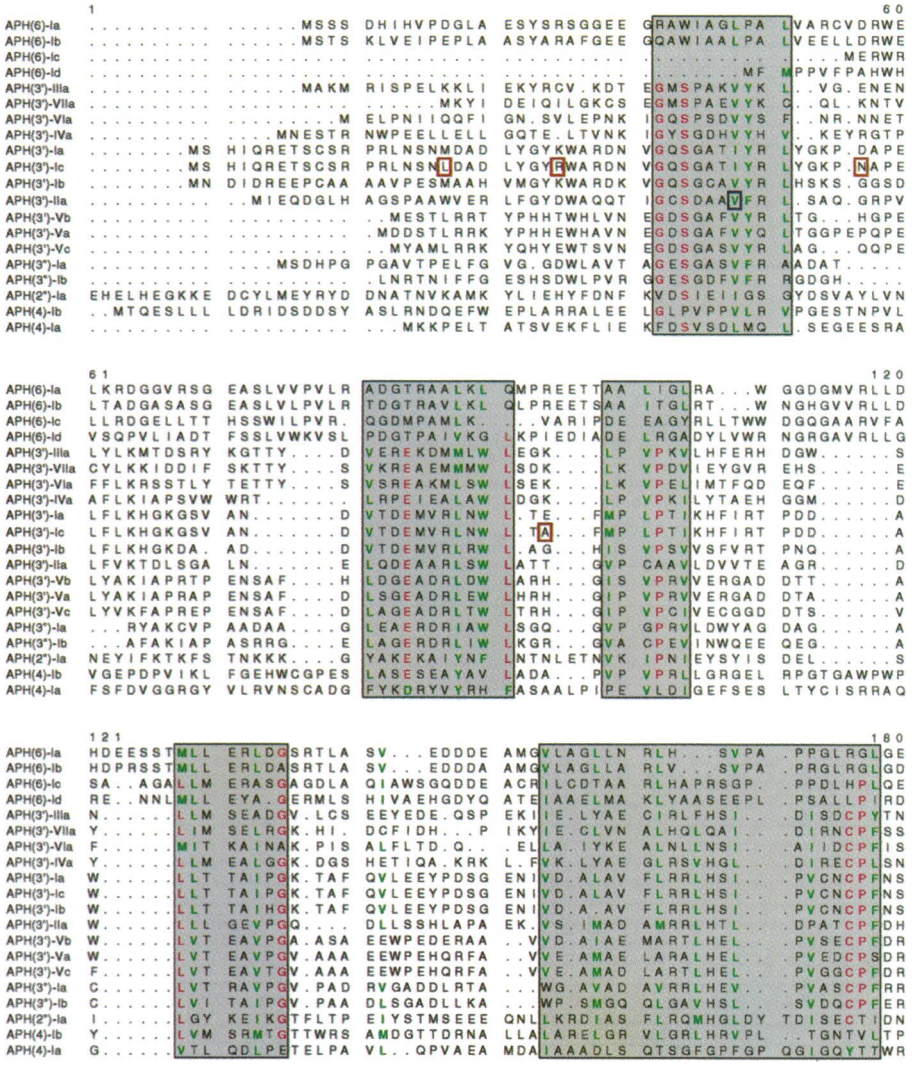

FIG. 9. Alignment of the APH family of enzymes. Alignment of protein sequences was performed by the Pileup Multiple Sequence Analysis Program (see above). Key: red, all 20 amino acids are identical; pink, at least 14 of the 20 amino acids are identical; green, at least 14 of the 20 amino acids are in one the similarity groups (C, Y), (D, E), (K, R), (F, L, W, Y), and (I, L, V, M).

[AAC(6′)-III profile], high-level resistance and increased enzyme activity were selected after five passages. The authors suggested that this resistance arose from alterations in the expression of a cryptic or poorly expressed chromosomal gene (31).

We have extended these findings through primer extension analysis to determine the start site and the amount of mRNA produced in susceptible and a resistant *Serratia* strains. The data clearly showed that abundant mRNA was produced in a resistant strain, whereas no mRNA was observed in the susceptible strain (91). The primer extension analysis also allowed the identification of the promoter region of the *aac(6′)-Ic* gene. The sequence of the −35 region is unusual (**CTTTTT**) and overlaps a large palindromic sequence. This region may represent the operator region where a repressor binds. We propose a model whereby an unlinked repressor binds to the operator region which is present in susceptible *Serratia* strains (91).

Southern analysis of clinical isolates which were susceptible or which expressed an AAC(6′)-I or AAC(6′)-III resistance profile showed that some *Serratia* strains which express an AAC(6′)-I resistance profile have altered restriction maps in the 5′ region of this gene (91). These data suggested that DNA rearrangements had occurred. It is likely that these rearrangements introduce a strong, constitutive promoter 5′ to the *aac(6′)-Ic* gene (91). We hypothesize that expression of the AAC(6′)-I and AAC(6′)-III AGRPs, encoded by the *aac(6′)-Ic* gene, can be due to a variety of mutations (91). (i) Mutation of a *trans*-acting negative regulator, which normally binds to the operator sequence, would allow transcription from the *aac(6′)-Ic* promoter; (ii) insertion of sequences upstream or within the 5′ coding region of the *aac(6′)-Ic* gene, would allow high level expression from an alternative, unregulated promoter; or (iii) point mutations which create a new promoter. Poor expression from a new promoter could result in low-level expression of the *aac(6′)-Ic* gene, which is defined as an AAC(6′)-III AGRP (resistance, by National Committee for Clinical Laboratory Standards-like criteria, to 2′-*N*-ethylnetilmicin only).

Since the chromosomal *aac(6′)-Ic* gene is found in all *S.*

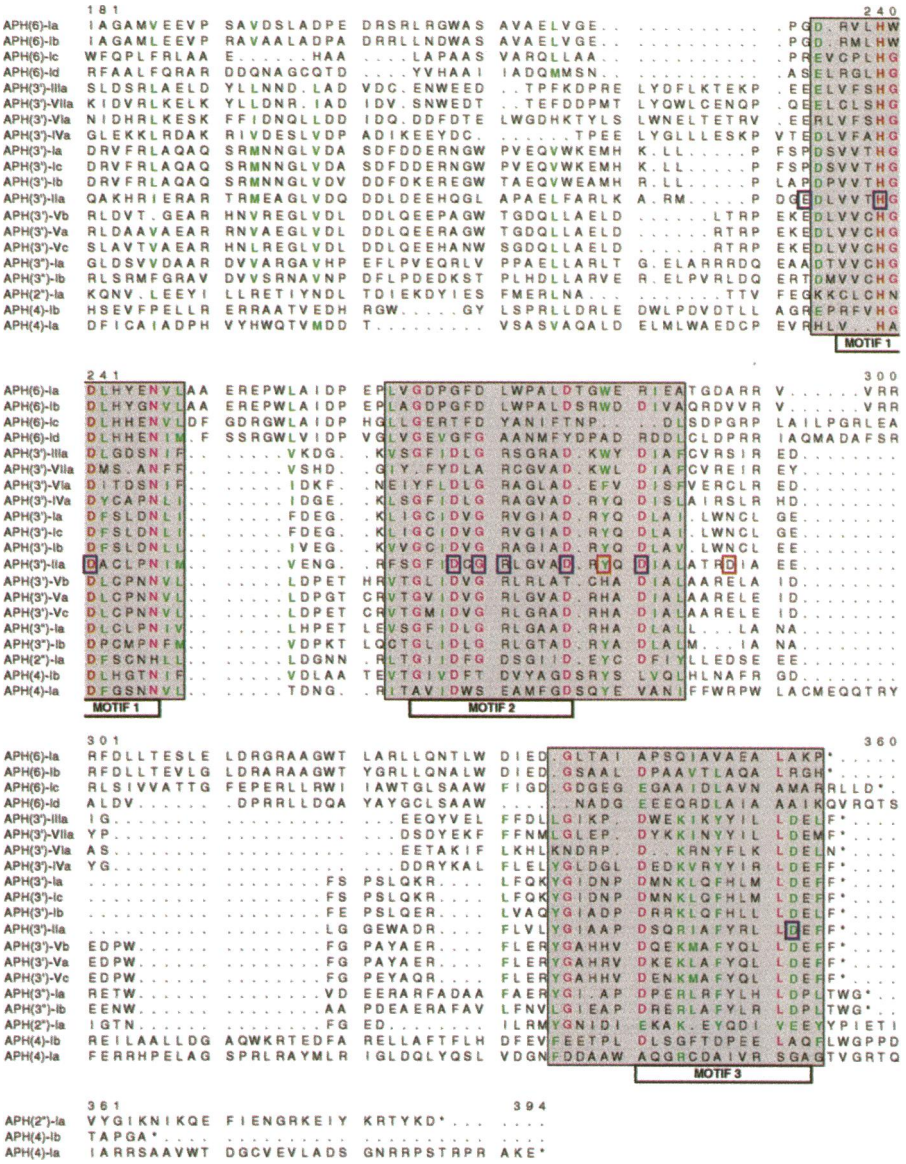

FIG. 9—*Continued.*

marcescens strains, it may encode an intrinsic but as yet undescribed acetyltransferase, which may play a role in primary metabolism (91). Although RNA analysis of the steady-state level of mRNA revealed little, if any, *aac(6′)-Ic* mRNA synthesis in a susceptible strain (91), it is possible that this gene is expressed only at a specific stage in the cell cycle. Determination of the cellular role of the AAC(6′)-Ic protein should reveal interesting information on the evolution of this resistance gene.

The chromosomal gene in a susceptible *Serratia* strain may represent an early stage in the development of an aminoglycoside-modifying enzyme. It is clear that mutations within, adjacent to, or regulating the *aac(6′)-Ic* gene are necessary for expression of resistance in individual *Serratia* strains. The ability to obtain expression of the transcriptionally silent *aac(6′)-Ic* through a variety of mechanisms explains why *Serratia* strains can so rapidly become resistant to aminoglycosides. It is possible that further selection, through the use of tobramycin, netilmicin, or amikacin, may result in the eventual mobilization of this gene via association with plasmids or transposons.

A second gene, *aac(2′)-Ia*, also appears to be tightly regulated. Primer extension analysis of susceptible *Providencia stuartii* isolates showed that the *aac(2′)-Ia* gene is transcribed at low levels (78). The identification of the *aac(2′)-Ia* promoter revealed striking similarities to the *aac(6′)-Ic* promoter at both the −10 and −35 regions. The −35 region of the *aac(2′)-Ia* promoter (**CTTTTT**) is identical to the −35 region of the *aac(6′)-Ic* promoter. The −10 region (**TATAAT**) is conserved at four of six bases when compared with the *aac(6′)-Ic* promoter. The unusual shared sequence at the −35 region suggests that both of these genes may be regulated by a novel mechanism that is common to both species.

To examine the regulation of the *aac(2′)-Ia* gene, we have isolated mutations that result in high-level expression of the

aac(2')-Ia gene (78). These mutations arise at frequencies of 10^{-6} to 10^{-7}. Introduction of a plasmid containing a transcriptional *aac(2')-Ia–lacZ* fusion into these mutants resulted in a large increase in β-galactosidase expression. These data suggest that the mutations resulting in a high-level AAC(2')-I resistance profile are affecting a *trans*-acting factor which regulates the *aac(2')-Ia* promoter. Interestingly, introduction of an *aac(6')-Ic–lacZ* fusion into these mutants also resulted in a large increase in β-galactosidase expression, relative to the same fusion in an isogenic, susceptible strain. These data provide compelling evidence that a common factor can control the expression of both the *aac(2')-Ia* and *aac(6')-Ic* genes.

CELLULAR LOCALIZATION OF AMINOGLYCOSIDE-MODIFYING ENZYMES

The intracellular location of the aminoglycoside-modifying enzymes may have a role in determining the level of resistance of the organism. If an enzyme is directed to the wrong cellular location within the bacterial cell, it may inefficiently inactivate the aminoglycoside. It has been proposed that since aminoglycosides inhibit the function of the bacterial ribosome, a cytoplasmic component, the modifying enzymes are present within the cytoplasm. An amikacin 3'-phosphotransferase has been suggested to be cytoplasmically located in *E. coli* (71). Similarly, the sequence of the first 6 amino acids of the AAC(3)-IVa protein showed that processing did not occur and that therefore the enzyme was likely to be cytoplasmically located (9). The cytoplasmic location of these enzymes is likely to be inefficient because a certain percentage of aminoglycoside molecules present in the cytoplasm would escape modification and inhibit protein synthesis. A more efficient mechanism would be for modification of the aminoglycoside to occur before it enters the cytoplasmic compartment.

In contrast to these studies, the ANT(3")-Ia protein has been reported to be located in the periplasm (37). In this study, the ANT(3")-Ia protein was released upon osmotic shock. Furthermore, some maxicell preparations showed the presence of a larger band, which was proposed to be the unprocessed precursor of the periplasmic enzyme (37).

Bacterial signal sequences are composed of 20 to 30 amino acids at the N terminus (18). This region is involved in the export of proteins across the cytoplasmic membrane and into the periplasmic space. The signal sequences are characterized by several positively charged amino acids followed by a hydrophobic stretch of amino acids and a peptidase cleavage site consensus sequence. The cleavage site consists of small amino acid residues at positions −3 (A, G, S, V, L, I) and −1 (A, G, S) preceding the cleavage site and a helix breaker between positions −6 and −4 (18). We have compared the amino-terminal sequences of the aminoglycoside-modifying enzymes and have identified prominent signal sequences in many of the AAC(3) family of proteins and in several of the AAC(6') enzymes (78a). Signal sequences are not found in the APH family of enzymes (78a). Although the amino-terminal ends of many of the AAC(3) proteins have not been determined by direct protein sequence analysis, at least two positively charged amino acids and a long hydrophobic stretch of amino acids are observed in the putative amino-terminal portion of most of the AAC(3) proteins. A consensus cleavage site (Fig. 7, positions 58 to 62) is also apparent in many of the AAC(3) enzymes (e.g., −6 [P]; −5 [Q]; −3 [V, L, I]; −1 [A, G, S]).

A prominent signal sequence has also been observed in at least one of the AAC(6') enzymes. DNA sequence analysis of the *aac(6')-Ib* gene revealed that the β-lactamase signal sequence was fused to the coding region of the *aac(6')-Ib* gene (102). To directly test the effect of a signal sequence, we fused the *aac(6')-IIa* gene, which lacks a signal sequence, in frame to DNA encoding the β-lactamase signal sequence. The MICs for *E. coli* containing this hybrid protein were eightfold higher than those for cells containing only the native AAC(6')-IIa protein (77). The cells containing the fused protein may be more resistant because the protein is now localized to the periplasm, although it is also possible that the amino-terminal sequences act by stabilizing the AAC(6')-IIa and AAC(6')-Ib proteins. These results suggest that the cellular location of the modifying enzyme may be important in determining resistance levels.

CONCLUSIONS AND PROSPECTS

Recent advances in the fields of bacterial aminoglycoside resistance and the molecular mechanisms involved are summarized below. The results of these studies have also provided general findings regarding the molecular biology of these modifying enzymes.

(i) DNA hybridization studies documenting the worldwide dissemination of aminoglycoside resistance genes have revealed that, for many enzymes, there are several genes encoding the same function. In many cases dissemination is aided by the integration of these genes into an integron, first observed in Tn*21*. This integration may help to explain the diverse combinations of resistance mechanisms that are found in many recent clinical isolates, which were not prevalent 10 years ago. For example, the prevalence of the AAC(6') genes in combination with other resistance genes has increased greatly in recent years.

(ii) Although it is likely that some of the genes encoding aminoglycoside-modifying enzymes originated from aminoglycoside-producing organisms, recent evidence has shown that others originated from chromosomal genes involved in cellular metabolism. New evidence on the regulation and expression of the *aac(6')-Ic* gene has been critical to understanding why *Serratia* strains can so rapidly become resistant to aminoglycosides.

(iii) Several of these proteins contain a putative signal sequence. We have shown that the presence of a signal sequence on the AAC(6')-IIa protein has a large effect on the level of resistance that is observed.

(iv) Proteins of a particular class are related. Examination of the relatedness of families of proteins and specific mutational analysis has allowed the determination of key sites for the interaction of aminoglycosides with the modifying enzymes. Our studies of the AAC(6')-Ib and AAC(6')-IIa enzymes (77), as well as those of Kocabiyik and Perlin (44) and Blázquez et al. (8), have shown that single-amino-acid changes can dramatically alter the substrate profile of an enzyme.

Recent results have had a significant impact on our present knowledge of the molecular biology and structure-function relationships of the bacterial acetyltransferases. The data have dramatically changed our understanding of the origin, evolution, and dissemination of these genes. In addition, they provide insight into which new aminoglycoside resistance mechanisms may arise in the future, how fast they can be disseminated, and, potentially, how aminoglycoside usage can overcome some of the problems. Lastly, X-ray crystallization studies of the structures of these enzymes could reveal precisely where the substrate interacts and

what changes in the enzymes may potentially allow the emergence of new resistance profiles. We anxiously await the enhanced understanding that this detailed level of structure will bring in the next decade.

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